Regulation of Protein Degradation by ADP-Ribosylation

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REGULATION OF PROTEIN DEGRADATION BY ADP-RIBOSYLATION

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by
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Protein quality control is essential for cellular homeostasis. The Ubiquitin-Proteasome System (UPS) is responsible for the regulated breakdown of intracellular proteins. All proteins are not degraded at the same rate in the cell. For instance, global protein turnover rates in mammals change with an average time between 1-2 days. On the other hand, a handful of proteins such as myelin exhibits limited turnover for months or even years. The UPS recycles most of the short-lived proteins in a time range from minutes to days depending on their localization and post-translational modifications. The post-translational modification, poly-ADP-ribosylation has an estimated half-life of only 1-6 min.

My thesis research aimed to reveal how the substrates of Tankyrase are degraded rapidly. Using Drosophila melanogaster, I described the role of Iduna E3 ubiquitin ligase in the regulation of Axin and Tankyrase proteolysis. I found that Iduna controls the proliferation of stem cells in the Drosophila midgut. Using a MS-based approach, I identified lysine 598 as an ADP-ribose acceptor site in Drosophila Tankyrase and showed that Tnks$^{K598A}$ adult flies live significantly shorter than control flies. Tnks$^{K598A}$ adult flies also reduce their flight, climbing abilities, global protein poly-ADP-ribosylation, and the activation of JNK signaling with age. Furthermore, I demonstrated that the ubcD1 ubiquitin-conjugating enzyme enhances the ubiquitin ligase activity of Iduna. Finally, I proposed a model by which poly-ADP-ribosylation brings together Tankyrase, target proteins, E2 ubiquitin-conjugating enzymes, E3 ubiquitin ligases and 26S proteasomes to accelerate the breakdown of target proteins.
My work addresses the general question of how proteins can be rapidly turned over, focusing on the role of Tankyrase-mediated poly-ADP-ribosylation. This work provides novel mechanistic insights into the regulation of protein quality control. Ultimately, these results may be useful to guide the development of new therapies.
Dedicated to lovely my family (Sevgili Aileme)
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LIST OF ABBREVIATIONS

AD — Alzheimer’s Disease
hNUDT16 — human Nudix type motif 16
AIF — apoptosis inducing factor
APC — adenomatous polyposis coli
DUB — deubiquitinating enzyme
pH3 — phospho-Histone 3
si-RNA — small- RNA
SDS-PAGE — sodium dodecyl sulfate polyacrylamide gel electrophoresis
r-RNA — ribosomal Ribonucleic acid
ARC — akyrin repeat clusters
ALS — amyotrophic lateral sclerosis
cADPR — cyclic ADP-ribose
ADP-r — ADP ribose
CRISPR — clustered regularly interspaced short palindromic repeats CKI
— Casein Kinase I
E1 — ubiquitin-activating enzymes
E2 — ubiquitin-conjugating enzymes
E3 — ubiquitin ligases
EC — enterocyte
EE — enteroendocrine
EB — enteroblast
FTD — frontotemporal dementia
GPCR — G-protein-coupled receptors

hnRNP A1 — heterogeneous nuclear riboprotein A1

ISC — intestinal stem cell

Na — nicotinic acid

Nam — nicotinamide

NAD+ — Nicotinamide adenine dinucleotide

NMDA — N-methyl-D-aspartate receptor

NMNAT — Nicotinamide mononucleotide adenylyltransferase

MAR — mono-ADP-ribose

MS — mass spectrophotometry

PAR — Poly-ADP-ribose

PARG — Poly-ADP-ribose glycohydrolase

PARP — Poly-ADP-ribose polymerase

PARsylate — to poly-ADP-ribosylate

PARsylation — poly-ADP-ribosylation

PI31 — Proteasome inhibitor 31 kDA

PBM — PAR-binding motif

PTRE1 — Proteasome regulator 1

GSK3β — glycogen synthase kinase 3β

RNF146 — Ring finger protein 146

RFLP — restriction fragment length polymorphism

SAM — a sterile-alpha motif

TNKS — Tankyrase
TDP-43 — TAR DNA binding 43kDA

TGA— Triglycerides

TARG1— Terminal ADP-ribose glycohydrolase 1

TBM — Tankyrase-binding motif

UPS — Ubiquitin-Proteasome System
1 INTRODUCTION

1.1 Protein quality control and the Ubiquitin-Proteasome System

Proteins are essential to execute the diverse cellular functions to maintain cell and organismal viability. The proper balance between protein biogenesis and protein degradation is crucial for normal cellular function. All living cells must carry out a rigorous quality control process in which both nascent and newly synthesized proteins are examined to maintain proper cellular functions. Thus, this process is crucial to protect cells against the pathological accumulation of unfolded, misfolded and damaged proteins (Narayan et al., 2014).

Protein quality control can deliver damaged proteins to the lysosomal proteolysis pathway or the ubiquitin–proteasome system (UPS) for their degradation in eukaryotic cells (Murata et al., 2009; Xie et al., 2010; Glickman and Ciechanover, 2002). Lysosome-mediated protein degradation is a bulk process that involves the uptake of extracellular proteins, cytoplasmic organelles, or cytoplasmic proteins in the membrane-bound vesicles fusing with the lysosome. Eventually, engulfed proteins are exposed to digestive enzymes inside these membrane-bound vesicles (Rousseau and Bertolotti, 2018; Narayan et al., 2014). On the other hand, the UPS is highly selective for individual proteins tagged for destruction, and it mediates the degradation of most short-lived proteins that control cell cycle, cell survival, cellular metabolism, transcription, DNA repair, and apoptosis (Rousseau and Bertolotti, 2018; Narayan et al., 2014). The proteasome is also essential for amino acid homeostasis (Rousseau and Bertolotti, 2018). Finally, the proteasome particles in the immune system generate major histocompatibility complex class I-presented peptides by breaking down the proteins derived from intracellular pathogens (Murata et al., 2009; Xie et al., 2010; Glickman and Ciechanover, 2002). Not only the diversity but also the complexity of the proteasome functions may explain
why aberrant proteasome function is often linked to the pathogenesis of human diseases, such as cancer, auto-inflammatory disorders and neurodegenerative diseases (Xie et al., 2010; Glickman and Ciechanover, 2002).

1.1.1 The composition of the UPS and the ubiquitin pathway

There are two types of signals for the proteasome-mediated protein turnover. The first one is ubiquitylation, which most commonly is in the form of lysine 48-linked poly-ubiquitin chains. The second is the presence of unstructured regions in proteins that can act as initiators for their degradation (de Bie and Ciechanover, 2011).

The UPS contains enzymes to modify target proteins by attaching multiple ubiquitin chains for their degradation. Ubiquitin (Ub) is a highly conserved 76 amino acid polypeptide that is usually attached to lysine residues on target proteins (Glickman and Ciechanover, 2002; de Bie and Ciechanover, 2011; Hershko and Ciechanover, 1998). Conjugation of target proteins with poly-Ub chains is a multi-step reaction that is catalyzed by distinct enzymes. These include the ubiquitin activating enzymes (E1s), ubiquitin conjugating enzymes (E2s), and ubiquitin ligases (E3s). After poly-ubiquitylation, target proteins are destructed by a multi-subunit protease termed the 26S proteasome (Murata et al., 2009; Xie et al., 2010; Glickman and Ciechanover, 2002).
Figure 1.1 The Ubiquitin-Proteasome system-mediated protein degradation. Ubiquitylation is a crucial step to tag substrates for degradation by the 26S proteasome. Conjugating substrates with poly-ubiquitin involves three distinct classes of enzymes, E1, E2, and E3s that work in concert to attach distinct ubiquitin chains. The ubiquitin-activating enzyme, E1, uses ATP to activate ubiquitin to transfer to the E2, ubiquitin-conjugating enzyme. The last step catalyzed by the E3 ubiquitin-ligase taking ubiquitin to link it to a lysine residue on the substrate protein. The specificity of degradation is largely mediated by E3 ubiquitin-ligases, which are abundant and recognize one or a few specific proteins. Following the ubiquitylation, the substrate is degraded by the 26S proteasome. The figure was adapted from (Vilchez et al., 2014).
The diagram illustrates the ubiquitin-proteasome system (UPS), a cellular system responsible for the degradation of proteins through a series of enzymatic steps:

1. **ATP** → **AMP + PPi**
2. **Ub** attaches to **E1** (Ub-activating enzyme).
3. **Ub** is transferred to **E2** (Ub-conjugating enzyme).
4. **Ub** then attaches to **E3** (Ub-ligase).
5. **Ub** is transferred to the **Target protein** and marked for degradation.
6. **Ub** is removed from the **Target protein** by the **20S Core particle** (α-Ring α1-α7, β-Ring β1-β7).
7. The **19S regulatory particle** (Lid Rpn 3,5,9,11,12, Base Rpt1-6, Rpn1,2,10, Hetero PA28 α, β, Homo PA28 γ, Bim10/PA200)并与 **20S core particle** (26S Proteasome) interacts to degraded the **Target protein**.
8. Peptides are produced from the degraded proteins.

The **Quality control** (Misfolded/damaged proteins), **Stress response** (Oxidized proteins), **Signal transduction** (Nf-κB), **Immunity** (Presentation of antigens), and **Cell cycle** (p21, p53) are regulated by the UPS.
In the first step of ubiquitylation, one of the E1’s activates ubiquitin in an ATP-dependent manner that generates a high-energy thiol-ester intermediate. Next, one of the E2s receives the activated ubiquitin from the E1 and forms a similar thiol-ester ubiquitin intermediate before binding to one of the E3s. Finally, the E3 ubiquitin ligase binds to a specific substrate and takes the activated ubiquitin from the E2 to transfer that to the target protein (Finley, 2009). Therefore, E3 ubiquitin ligases are key to substrate specificity in the UPS. The subsequent additional ubiquitin to lysine residues on previously conjugated enzyme forms the poly-ubiquitin chain that is recognized by the 26S proteasome.

The 26S proteasome is the major non-lysosomal protease devoted to protein breakdown in eukaryotic cells. This is a sophisticated 2.5 MDa multimeric self-compartemntalized protease complex. It contains two sub-complexes, the 20S proteasome, which forms the proteolytic core particle, and the 19S regulatory particle, which recognizes poly-Ub targets and delivers them in an ATP-dependent manner to the core particle (Murata et al., 2009; Xie et al., 2010; Glickman and Ciechanover, 2002). The 20S is a cylindrical particle that is formed by axial stacking of four hetero-heptameric rings: Two outer α-rings and two inner β-rings, each comprising seven structurally similar α- and β- subunits, respectively. β-rings form a proteolytic chamber and α-rings serve as a gate for entry into the chamber (Murata et al., 2009; Xie et al., 2010; Glickman and Ciechanover, 2002). The 19S proteasome contains six ATPase subunits (Rpt1-6) that are organized in a hexameric ring and facilitate gate-opening and substrate unfolding by using ATP (Murata et al., 2009; Xie et al., 2010; Glickman and Ciechanover, 2002).
1.1.2 The regulation of the UPS

Protein breakdown by the UPS is controlled at different levels. It involves both substrate ubiquitylation as well as regulation of proteasome assembly and activity. The attachment of ubiquitin to lysine residues allows for branched poly-ubiquitin chains. This branching is tightly regulated and results in different biological processes, such as protein degradation by the 26S proteasome when ubiquitinylation takes place in lysine 48, and modification of signaling cascades when lysine 63 is ubiquitylated (de Bie and Ciechanover, 2011).

Alternative activators, various interacting proteins and post-translational modifications (PTMs) can regulate the assembly and the activity of the 26S proteasome (Rousseau and Bertolotti, 2018). While the majority of proteasomes in eukaryotic cells are composed of 20S and 19S core particles to form the 26S proteasome, populations of proteasomes can be heterogeneous in a tissue ( Förster et al., 2005).

PTMs of the proteasome can regulate protein degradation. Protein phosphorylation by various kinases and phosphatases can promote or repress the activity of the proteasome (Guo et al., 2017; Rousseau and Bertolotti, 2018). Serine 120 of Rpt6 was first demonstrated to be phosphorylated by cAMP-dependent protein kinase A (PKA) and dephosphorylated by protein phosphatase 1-gamma (Satoh et al., 2001; Pereira and Milk, 1990; Asai et al., 2009; Rousseau and Bertolotti, 2018). PKA-mediated phosphorylation of Rpt6 regulates the 26S proteasome, possibly by promoting the binding Rpt6 to α-2 subunit of the 20S (Satoh et al., 2001).

Tankyrase (TNKS)-mediated ADP-ribosylation is another PTM that regulates 26S proteasome assembly and activity. PI31 (Proteasome inhibitor 31 kDA) was initially identified based on its ability to inhibit 20S proteasome activity in vitro (Chu-Ping et al., 1992; Zaiss et al., 1999). TNKS ADP-ribosylates PI31, which in turn alters the binding-affinity of PI31 for 20S
proteasome alpha subunits and 19S assembly chaperons. As a consequence, PI31-ADP ribosylation stimulates 26S proteasome assembly and activity (Park and Steller, 2013). *In vivo* studies of PI31 in *Drosophila* demonstrated that PI31 is an activator of the 26S proteasome (Bader et al., 2011). Loss of *PI31* causes accumulation of poly-ubiquitylated proteins in *Drosophila* (Bader et al., 2011).

Moreover, orthologs of PI31 in yeast and plants have been shown to stimulate proteasome activity and protein breakdown (Yashiroda et al., 2015; Yang et al., 2016). Our lab has recently found that PI31 plays a role in proteasome transport and is required for synaptic structure and function (Liu et al., 2019). PI31 directly couples proteasomes to dynein light chain proteins (DYNLL1/2). Depletion of *PI31* inhibits proteasome motility in axons and disrupts synaptic protein homeostasis, structure, and function (Liu et al., 2019).

The proteasome can be specialized in certain tissues through the incorporation of specific subunits. For example, the immune-proteasome has four additional β-subunits, and the thymoproteasome contains a thymus-specific β5-t subunit (Kloetzel, 2004; Tanaka and Kasahara, 1998; Murata et al., 2009). These alternative proteasomes have important roles in adaptive immunity to present antigens. On the other hand, mammalian and *Drosophila* testis have specific isoforms of 20S proteasome subunits (Belote et al., 1998; Ma et al., 2002; Zhong and Belote, 2007).
Figure 1.2 Model for increased proteasome assembly upon TNKS-mediated ADP-ribosylation of PI31. PI31 is regulated by TNKS-mediated ADP-ribosylation, which alters the binding affinity of PI31 for 20S proteasome alpha subunits and 19S assembly chaperones. As a consequence, PI31-ADP-ribosylation promotes the 26S proteasome assembly and activity. The figure was adapted from (Park and Steller, 2013).
Figure 1.3 Model for PI31-mediated transport of proteasomes in axons. Proteasomes can move rapidly along microtubules to fulfill dynamic local demands for protein degradation in different cellular compartments. PI31 is an adaptor protein for proteasome transport in *Drosophila* axons. PI31 directly binds to proteasomes with dynein light chain proteins (DYNLL1/2). p38-MAP kinase-mediated PI31 phosphorylation enhances binding of PI31 to DYNLL1/2. The figure was adapted from (Liu et al., 2019).
1.2 ADP-ribosylation as a post-translational modification

ADP-ribosylation is a strikingly dynamic and reversible PTM, in which ADP ribose (ADP-r) units are attached to acceptor proteins by ADP-r transferases. The first ADP-ribose transferase was discovered as a bacterial toxin (Schreiber et al., 2006). Cholera and diphtheria toxins are ADP-ribose transferases, inhibiting or enhancing the protein synthesis or GPCR signaling in the host cells, respectively (Ruf et al., 1996; Oliver et al., 2004; Hassa et al., 2006; Perina et al., 2014). In eukaryotes, ADP-ribosylation affects many cellular processes. Therefore, aberrant regulation of ADP-ribosylation causes human diseases including acute central nervous system disorders, inflammatory diseases, and cancer (Palazzo et al., 2015; Schreiber et al., 2006).

Poly-ADP-ribosylation (PARsylation), on the other hand, is an endogenous reversible PTM of proteins that is mediated by poly-ADP-ribose polymerases (PARPs). PARPs generate negatively charged large polymers by adding ADP-r units to the acceptor proteins. Glu, Asp, Lys, Arg, Cys, Thr, or phosphorylated Ser are the amino acid residues that can accept the poly-ADP-ribose moieties (Daniels et al., 2014; DaRosa et al., 2018; Martello et al., 2016; Zhang et al., 2013).

Nicotinamide adenine dinucleotide (NAD+) is a co-substrate of PARPs to generate ADP-r polymers on protein acceptors. PARPs transfer multiple ADP-r units to their substrates forming both elongated and also branched poly-ADP-ribose modifications (Schreiber, 2006; Ame, 2004). Therefore, in contrast to several other PTMs, covalently attached PAR molecules on an acceptor protein differ greatly in size up to several hundred ADP-ribose residues with branching and large negative charges (Miwa and Masutani, 2007; Miwa et al., 1979; Hayashi et al., 1983). PARsylation can influence protein fate through several mechanisms, including a direct effect on
protein activity, recruitment of binding partners that recognize PAR or by affecting protein turnover (Guettler et al., 2011).

1.2.1 The roles of poly-ADP-ribose polymerases

At this time, 17 PARP members have been identified from genome sequencing (Schreiber et al., 2006). PARP1 is the first investigated PARP, that is involved in DNA repair following by its activation in response to single and double strand DNA breaks (Schreiber et al., 2006). PARP1 activity undergoes an over 500-fold increase upon its binding to DNA strand breaks (Eustermann et al., 2015; Nottbohn et al., 2007; Fan et al., 2018). During DNA damage repair, PARP1-induced auto-PARsylation acts as a molecular scaffold to recruit DNA damage repair proteins to the close proximity of the DNA strand breaks. PARP1 also poly-ADP-ribosylates histone tails, causing chromatin relaxation. This allows the recruitment of several chromatin remodelers through their binding to PAR, which further relax chromatin to facilitate DNA repair (Chaudhuri and Nussenzweig, 2017). As a result, DNA damage is repaired within several minutes. Hence, Parp1 mutant animals have different vulnerability spectrum to the genotoxic mutagens (Menissier et al., 2003; Miwa and Masutani, 2007).
ADP-ribose transferases (ARTs) use NAD+ to modify their target proteins. ADP-ribosylation is a very dynamic and reversible post-translational modification. Target proteins can be mono-ADP-ribosylated or poly-ADP-ribosylated by ARTs. ADP-ribosylation has an impact on protein fate through several mechanisms including changing localization, activity, stability, protein interactions and complex assembly.

Figure 1.4 ADP-ribose transferases (ARTs) use NAD+ to modify their target proteins.
Figure 1.5 ADP-ribosylation generates a scaffold for DNA damage repair proteins. PARP1-induced auto-PARsylation acts as a molecular scaffold to recruit DNA damage repair proteins to the close proximity of the DNA strand breaks. Furthermore, PARP1 PARylates histone tails, which causes chromatin relaxation. This allows the recruitment of several chromatin remodelers through their binding to PAR, which further relax chromatin to facilitate DNA repair. The figure is adapted from (Beneke et al., 2012).
PARP1-dependent PARsylation also controls the nuclear localization of several proteins such as pyruvate kinase isoform M2, a glycolytic enzyme that converts phosphoenolpyruvate to pyruvate, in EGFR-mutant cancer cells (Li et al., 2016). PKM2 is not a PARsylation target of PARP1, however, PKM2 has a canonical PAR-binding motif. PAR binding is required for PKM2 nuclear retention. That is crucial for glycolysis and tumor growth. It is also shown that PAR level correlates with nuclear localization of PKM2 in EGFR mutant cells (Li et al., 2016). Therefore, inhibition of PARP1-dependent PAR generation suppresses the growth of EGFR mutant cancers.

Nuclear PARP1 also facilitates ribosomal biogenesis in *Drosophila* (Boamah et al., 2012). PARP1 becomes auto-PARsylated upon each act of transcription start within r-DNA gene. Auto-PARsylated PARP1 serves as a chaperoning machine during whole cycle of ribosome maturation in nucleus. The dynamic PARP1-mediated PAR scaffold brings a subset of nucleolar proteins to the proximity of precursor r-RNA and coordinates the order of events including r-RNA processing, modification and loading of subsets of ribosomal proteins to mature pre-ribosome in *Drosophila* (Boamah et al., 2012). Depletion of *Parp1* leads to the loss of PAR-binding proteins from nucleoli, which disrupt processing, modification and folding of ribosomal RNA in *Drosophila* (Boamah et al., 2012).

*Tnks (Parp5)* has two isoforms in mammals. Tnks proteins contain a PARP domain at the C-terminus, and dimerization of the catalytic domain is required for TNKS activity (Fan et al., 2018). Immediately upstream of its catalytic domain, there is a SAM, a sterile-alpha motif, domain required for the oligomerization of TNKS (Smith et al., 1998; De Rycker and Price, 2004). The basal activity of the isolated TNKS catalytic domain is extremely low, approximately 1000-fold lower than PARP1 (Nottbohm et al., 2007). The activity is dramatically elevated by
the SAM domain-dependent polymerization (Nottbohm et al., 2007; Fan et al., 2018). The stimulation of Wnt signaling by TNKS1/2 is dependent on SAM domain-mediated oligomerization, which is important for both TNKS localization and substrate recruitment (Marino-Echarri et al., 2016; Fan et al., 2018). The polymerization of TNKS1/2 is inhibited by auto-PARsylation (De Rycker and Price, 2004). Finally, there is a large ankyrin repeat domain, containing conserved ankyrin repeat clusters (ARCs), which are known for substrate recognition and binding (Lehtio et al., 2013). For instance, ARC5 appears essential for TRF1 PARsylation, the release of TRF1 from telomeres and telomere elongation (Seimiya et al., 2004). Each substrate has a TNKS binding motif (TBM) with an RxxxxG consensus, which mediates binding to the ARC domain (DaRosa et al., 2018; Guettler et al., 2011).

TNKS1/2 play important roles in cellular pathways that are critical for cancer cell growth, including telomere cohesion and length maintenance, GLUT-4-mediated vesicle transport, the Wnt/β-catenin pathway, as well as apoptosis (Daniels et al., 2014). Studies on human Tnks1/2 knock out cell lines indicate that they are necessary to maintain telomere cohesion and mitotic spindle integrity (Bhardwaj et al., 2014). The quantitative proteome analysis of human TNKS double knockout cells demonstrates Notch1, 2, 3 receptors are the targets of TNKS (Bhardwaj et al., 2014).

Mutations in the immune adaptor protein 3BP2 that impair interaction with TNKS cause Cherubism, characterized by progressive, painless, bilateral swelling of the jaw during childhood. Loss of TNKS-mediated destruction of 3BP2 through Iduna is the underlying mechanism of the disease (Levaot et al., 2011; Guettler et al., 2011 Ueki et al., 2007).

In contrast to mammals, Drosophila has only a single Tnks gene. The overall similarity between Drosophila and human TNKS1/2 proteins is 79% (Wang et al., 2016). While double
knockout of Tnks1 and 2 lead to lethality in mice, Drosophila Tnks is not required for viability (Feng et al., 2014). TNKS is essential for Axin degradation in vivo but loss-of Tnks does not lead to obvious morphological defect in Drosophila (Feng et al., 2014; Yang et al., 2016; Wang et al., 2016). TNKS antagonizes the β-catenin destruction complex through ADP-ribosylation of Axin and APC2 (Kroy et al., 2016). Therefore, the Wnt pathway is regulated by ADP-ribosylation.

Genetic depletion of Tnks causes Axin elevation that blocks Wingless signaling in enterocytes and activates JAK-STAT signaling to promote intestinal stem cell proliferation in the Drosophila midgut (Wang et al., 2016). Conditional genetic inactivation of Tnks1/2 in mice results in a rapid decrease of Lgr5+ intestinal stem cells and promotes cell death in small intestinal crypts. As a result of intestine degeneration, increased mice mortality is indicated (Ye et al., 2018). On the other hand, TNKS can stimulate apoptosis in the Drosophila eye and wing by activating JNK signaling (Feng et al., 2018).

TNKS also has other PTMs but their impacts on TNKS are not well understood. Mitogen-activated protein kinase phosphorylates TNKS1 on its serine residues by after insulin or growth factor stimulation (Chi and Lodish, 2000). TNKS can be hydroxylated by hypoxia-inducible factor asparagine hydroxylase (FIH), although precise functions of these PTMs are not clear (Cockman et al., 2009). Finally, auto-PARsylation of TNKS provides a recognition signal for ubiquitylation and 26S-proteasome-mediated degradation through the Iduna (RNF146) (Gultekin and Steller, 2019; Callow et al., 2011; Zhang et al., 2011).

1.2.2 Regulation of ADP-ribosylation

As a highly dynamic and reversible PTM, poly-ADP-ribosylation has an estimated half live of only 1-6 min (Alvarez-Gonzales et. al., 1989). PAR glycohydrolase (PARG), Nudix type
motif 16 (NUDIX16) and NUDIX9 can remove PARsylation (Slade et al., 2011; Lin et al. 1997; Slade et al., 2011; Palazzo et al., 2015).

The opposing effects of PARPs and PARG in regulating protein PARsylation are important in a variety of cellular processes, including chromatin modeling, DNA repair, transcription regulation and cell death (Ahel et al., 2008; Andrabi et al., 2006; Frizzell et al., 2009; Sing et al., 2017). Impairments in the hydrolysis of PAR chains, therefore, involve in various human disorders such as cancer, neurodegeneration, oxidative stress, neural injury, and regeneration (Deng, 2009; Hanai et al., 2004; Martire et al., 2015; Brochier et al., 2015). Interestingly, PAR, some PARPs as well as PARG can be found in cytoplasmic stress granules and may regulate micro-RNA-dependent translational repression and mRNA cleavage (Gagne et al, 2008; Kotova et al., 2009; Leung et al., 2011). Mutations on RNA-binding proteins such as heterogeneous nuclear riboprotein A1 and TAR DNA binding 43kDA (TDP-43) enhance protein aggregation in mouse and fly models of familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Kim et al., 2013; Neumann et al., 2006). TNKS-mediated PARsylation modulates stress granule assembly, phase separation and neurotoxicity of ALS-related RNA binding proteins hnRNP A1 and TDP43 (McGurk et al., 2018; Duan et al., 2018).

There are also other enzymes that cleave mono-ADP-ribose moieties on target proteins. For instance, Terminal ADP-ribose glycohydrolase (TARG1) can remove mono-ADP-ribose on glutamate residues (Sharifi et al., 2013). Homozygous mutations of TARG1 are associated with severe neurodegeneration in human patients (Sharifi et al., 2013). The source of ADP-ribose units is NAD+, which has been shown in a variety of systems to be neuroprotective and increases health-span (Lehmann et al., 2016; Ocampo et al., 2013; Verdin, 2015; Wang et al., 2008; Zhang et al., 2016).
Figure 1.6 TNKS-mediated ADP-ribosylation largely stimulates ubiquitylation and substrate degradation by the 26S proteasome. TNKS is conserved from flies to human. Fly genome encodes a single *Tnks* gene. In contrast, mammalian cells have two TNKS isoforms. Both structure and amino acid composition of fly TNKS are similar to mammalian TNKSs. Ankyrin repeat clusters (ARCs) are responsible for recognizing and binding to the substrate proteins. α-sterile-alpha motif is required for TNKS oligomerization. Poly-ADP-ribose polymerase domain is the catalytic region of TNKS. TNKS makes a complex with Iduna E3 ubiquitin ligase. Then, TNKS binds to its target protein and ADP-ribosylates that by using NAD+. When the modification in the target protein is iso-or poly-ADP-ribose, Iduna E3 ubiquitin ligase is activated and ubiquitinylates the target protein. Finally, the poly-ubiquitinylated target protein is degraded by 26S proteasomes. The functional domain image of TNKSs is adapted from (Wang et al., 2016).
<table>
<thead>
<tr>
<th>Similarity</th>
<th>dTnks</th>
<th>hTNKS1</th>
<th>hTNKS2</th>
</tr>
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<tbody>
<tr>
<td>82%</td>
<td>ARC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>93%</td>
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<tr>
<td>73%</td>
<td>ARC3</td>
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<tr>
<td>92%</td>
<td>ARC5</td>
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<td></td>
</tr>
<tr>
<td>84%</td>
<td>SAM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>86%</td>
<td>PARP</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1181aa</td>
<td>1327aa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1166aa</td>
</tr>
</tbody>
</table>

Diagram showing the interaction of ADP-ribose, TNKS, substrate, NAD+, poly-ub, and 26S Proteasome with poly-peptides.
Depletion of NAD+ leads to failure to generate ATP, a major requirement for many cellular processes. Therefore, recycling and production of NAD+ is crucial.

NAD+ producing pathways include de novo synthesis from tryptophan, and a salvage pathway, which recycles nicotinamide and nicotinic acid from the diet. PARPs, c-ADP-ribose synthases and Sir tuins are general NAD+ consumers (Cantó et al., 2013). For instance, CD157 or Bst-1 is an ectoenzyme on Paneth cells in the mouse small intestine. Caloric restriction increases NAD+ levels (Guarente and Picard, 2005). Upon caloric restriction, elevated Bst1 is thought to promote stem cell proliferation through converting NAD+ to cyclic ADP-ribose (cADRP) (Yilmaz et al., 2012; Igarashi and Guarente, 2016).

Dietary nicotinic acid (Na) and nicotinamide (Nam) intake significantly alters NAD+ levels in organisms (Belenky et al., 2007; Elhassan et al., 2017). The salvage pathway is crucial to metabolize the dietary NAD+ precursors, Na and Nam. Nicotinamide mononucleotide adenyltransferase (NMNAT) is an essential enzyme, participating in the synthesis of NAD+ from Na or Nam. Genetic depletion of nmnat results in lethality in Drosophila. Interestingly, the salvage pathway-mediated NAD+ boost increases longevity in various model systems (Lin et al., 2000; McClure et al., 2012; Ocampo et al., 2013). Therefore, NAD+ metabolism is important for poly-ADP-ribosylation.

1.2.3 RNF146/Iduna E3 ubiquitin ligase

Iduna, an evolutionarily conserved protein, is a unique E3 ubiquitin ligase that specifically promotes the degradation of PARsylated proteins (Zhou et al., 2011; Wang et al., 2012; He et al., 2012; DaRosa et al., 2014; Zhang et al., 2011; Callow et al., 2011).
Under normal physiological conditions, Iduna is found as an inactive enzyme. Binding to TNKS mediated iso- or poly-ADP-ribosylated proteins is an allosteric activation signal of Iduna-dependent ubiquitylation and the 26S proteasome-mediated degradation (DaRosa et al., 2014). Iduna is a positive regulator of Wnt signaling since it promotes Axin poly-ubiquitylation and degradation. Depletion of zygotic Rnf146 with a translational blocking morpholino in Xenopus causes anteriorized development (Zhu et al., 2017). Conditional inactivation of Iduna promotes osteoclast activity and inflammation in mouse bone (Matsumoto et al., 2017a). On the other hand, Iduna depletion reduces proliferation of osteoblasts and promotes adipogenesis in the mouse skeleton (Matsumoto et al., 2017b).

TNKS and Iduna have received considerable attention as potential targets for cancer therapy. Overexpression of Iduna in non-small cell lung cancer enhances proliferation and invasion of tumors through the Wnt/β-catenin pathway (Gao et al., 2014). Furthermore, knock down of Tnks1/2 stabilizes the tumor suppressor PTEN, reduces AKT phosphorylation and inhibits cell proliferation and glycolysis in vitro and tumor growth in vivo. TNKS1/2 are upregulated and negatively correlated with PTEN expression in human colon carcinomas (Li et al., 2015). Finally, TNKS and Iduna are positive regulators of Hippo-YAP signaling identified through a CRISPR screen (Wang et al., 2016). YAP is an oncoprotein whose expression is elevated in several human cancers. Small molecule inhibitors of TNKS suppress the oncogenic of YAP through stabilizing angiomotin family proteins via TNKS-Iduna axis (Wang et al., 2015; Wang et al., 2016).

On the other hand, Iduna protects mice against glutamate excitotoxicity and stroke (Andrabi et al., 2011). It also preserves cells from environmental stress-induced cell death by scavenging PARs (Zhou et al., 2011; Andrabi et al., 2011; Gerö et al., 2014; Kang et al., 2011;
Wang et al., 2011; Xu et al., 2013). Iduna has a neuroprotective role against NMDA toxin induced cell death. NMDA induces PAR polymers in the nucleus. Accumulated PAR diffuses into cytoplasm that interacts with apoptosis inducing factor (AIF). That leads to AIF release from mitochondria outer membrane. AIF then enters to nucleus and causes DNA fragmentation and cell death. Over-expression of Iduna in the mouse brain prevents the interaction of PARs with AIF. This abrogates AIF-release and blocks neuronal cell death in mice (Andrabi et al., 2011). Iduna is also highly expressed in the brain of patients that suffers from Alzheimer’s Disease (AD) (von Rotz et al., 2005). Hence, Iduna may play a role for protection against stress-induced cell death and neurodegeneration.

1.3 The Wnt/β-catenin (Wingless) signaling pathway

Wnt/β-catenin (Wingless) signaling is an evolutionarily conserved pathway from invertebrates to vertebrates. This pathway has critical functions during embryonic development, stem cell self-renewal and differentiation, adipogenesis, neural maturation, as well as tissue and animal regeneration (Nusse and Clevers, 2017).

The key process in the canonical pathway is to control of β-catenin concentration by a destruction complex, which is composed of Axin/GSK3, APC and Casein Kinase I (Kimelman and Xu, 2006; Nakamura et al., 1998; Hart et al., 1998; Ikeda et al., 1998). When there is no Wnt ligand, the destruction complex associates with β-catenin and enhances the degradation of phosphorylated and poly-ubiquitylated β-catenin through the 26S proteasome (Zeng et al., 1997). On the other hand, if there is a Wnt ligand, it binds to Frizzled and LRP receptors on the cell membrane. β-catenin is then stabilized with its degradation complex on the cell membrane,
accumulates in the cytoplasm and finally trans-locates into the nucleus to induce expression of the pathway-associated genes (Lee et al., 2003; Zeng et al., 1997).

Axin is the central scaffold protein in the destruction complex. It binds the components of the complex through its different domains (Nakamura et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Zeng et al., 1997). Axin is present at a lower level than the other components of the complex and over-expression of Axin in cultured cells promotes the degradation of β-catenin (Lee et al., 2003). Hence, Axin is a rate-limiting factor of the pathway and its level is tightly controlled (Lee et al., 2003; Zeng et al., 1997). There is an Axin threshold in a time and tissue-dependent manner in *Drosophila*. To observe functional alterations in Wingless activity in the developing embryo of *Drosophila*, there should be Axin elevation more than its endogenous protein level. This threshold is 3-6 fold of endogenous Axin level to observe abnormal wing development. On the other hand, adult *Drosophila* midgut is more sensitive tissue to reduced Wingless signaling (Feng et al., 2014; Wang et al., 2016; Yang et al., 2016). It is therefore required to degrade the rate-limiting protein Axin through the UPS to prevent the attenuation of Wnt signaling (Li et al., 2012).

Axin degradation depends on the combined actions of TNKS poly-ADP-ribose polymerase and Iduna (Zhang et al., 2011). UPS-dependent degradation of Axin occurs in a specific temporal order. As an initial step, Iduna binds to TNKS but Iduna initially exists in an inactive state in the complex. Once Axin binds to the Iduna-TNKS complex through its TBM, TNKS ADP-ribosylates Axin by using NAD+. After Axin has iso- or poly-ADP-ribose, Iduna changes its confirmation to recognize and bind to Axin through its WWE domain and poly-ubiquitylates Axin. After the ADP-ribosylation and ubiquitylation, post-translationally modified Axin is rapidly degraded by the 26S proteasome (DaRosa et al., 2014; Wang et al., 2016a and
24b; Croy et al., 2016; Callow et al., 2011). This tight control suggests an important function for Iduna to regulate the Wnt/β-catenin pathway.

1.4 Intestinal stem cells in the adult Drosophila midgut

Adult stem cells are essential for maintaining basal tissue integrity and are a resource for tissue regeneration upon injury. Tissue homeostasis requires a tight control to coordinate the actions between the removal of old or damaged cells, and the production of new cells. The intestinal epithelium has a remarkable regeneration potential. The small intestinal epithelium of the mouse completely renews itself every 3-5 days (Barker et al., 2007; Barker et al., 2012).

The Drosophila adult midgut has similar structure and function with the mammalian small intestine (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Barker et al., 2007). Similar to the mammalian intestine, the fly midgut provides essential physiological functions, including food digestion, nutrient absorption and solid waste elimination, and displaying key endocrine, immune and metabolic roles (Perochon et al., 2018).

The Drosophila midgut has intestinal stem cells (ISCs) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Paracrine Wingless signaling controls self-renewal of Drosophila midgut stem cells (Perochon et al., 2018; Herr et al., 2012; Lin and Xi, 2008).

Under homeostatic conditions, reduced Wingless signaling causes over-proliferation of ISC in Drosophila. (Kramps et al., 2002; Wang et al., 2016 a, b; Tian et al., 2016; Gultekin and Steller, 2019). Non-cell autonomous mechanisms also orchestrate tissue remodeling in the Drosophila midgut, highlighting the importance of the stem cell niche as a sensor of organismal physiology. The JAK-STAT pathway can be non-cell autonomously activated to promote proliferation of stem cells in the Drosophila midgut (Gultekin and Steller, 2019; Herrera and
Bach, 2019; Zeidler et al., 2000; Zoranovic et al., 2013; Markstein et al., 2014; Zhou et al., 2013; Tian et al., 2016).

In response to genetically or pharmacologically induced stress, bacterial infection, and apoptosis, enterocytes and enteroblasts have been shown to express and secrete Unpaired (UPD) cytokines. Secreted UPDs then bind to the Domeless receptor on enteroblasts or intestinal stem cells to activate the JAK-STAT pathway in stem cells (Herrera and Bach, 2019; Ghiglione et al., 2002; Zhou et al., 2013; Xu et al., 2011). Moreover, other conserved signaling pathways including JNK, Hippo, EGRF, Hh, Dpp/BMP signaling have been shown to mediate damage or stress-induced intestinal regeneration in *Drosophila* (Jiang and Edgar, 2011; Naszai et al., 2015; Apidianakis et al., 2009; Jiang et al., 2009; Buchon et al., 2009; Shaw et al., 2010).
Figure 1.7 ADP-ribosylation activates the Wnt/β−catenin signaling pathway. When Wnt is `off`, the destruction complex, composed of Axin/GSK3, APC and Casein Kinase I associates with β−catenin and enhances the degradation of phosphorylated and poly-ubiquitylated β−catenin through the 26S proteasome (1). On the other hand, if Wnt is `on`, WNT ligand binds to Frizzled and LRP receptors on the cell membrane (2). β−catenin is then stabilized with its degradation complex on the cell membrane, accumulates in the cytoplasm and finally translocates into the nucleus to induce expression of the pathway-associated genes (3). On the other side, TNKS PARsylates Axin, the rate limiting protein of the Wnt pathway. Finally, PARsylated-Axin is poly-ubiquitylated by RNF146/Iduna for its turnover (4). The figure is adapted from (McCubrey et al., 2014).
Figure 1.8 Intestinal stem cells reside in the Drosophila midgut epithelium. A- Just as the mammalian intestine, the Drosophila midgut has several intestinal regions including the foregut, anterior midgut, the posterior midgut, and the hindgut. B- Drosophila intestine has stem cells (ISCs), which give rise to all intestinal compartments. ISCs give rise to daughter cells: undifferentiated enteroblasts (EBs) and terminally differentiated enterocytes (ECs) and enteroendocrine (EEs) cells. C- The Wingless pathway is necessary to maintain midgut homeostasis in Drosophila. Axin is a rate-limiting factor in the Wingless pathway. There is an Axin threshold to observe functional consequences of altered Wingless signaling in a time and tissue-dependent manner in Drosophila. Adult Drosophila midgut is more sensitive tissue to reduced Wingless signaling.
**A**

Diagram of the gut showing Foregut (fg) and hindgut.

**B**

Diagram showing EC, EB, EE, and ISC cells in *Drosophila* tissue.

**C**

<table>
<thead>
<tr>
<th>Drosophila tissue</th>
<th>Axin threshold (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>Wing imaginal discs</td>
<td>3-9</td>
</tr>
<tr>
<td>Adult midgut</td>
<td>≥ 2</td>
</tr>
</tbody>
</table>
1.5 Relevance to this thesis

The UPS degrades most of the short-lived proteins in the cell. The rates of proteolysis often correlate with cellular functions, cellular localization as well as post-translational modifications (PTM) of a protein. Poly-ADP-ribosylation is a highly dynamic and reversible PTM, affecting protein activity and stability. For instance, poly-ADP-ribosylated TRF1, a negative regulator of telomere length localized on the telomeres, has not been detected in vivo, owing to its rapid degradation (Ye and de Lange, 2004).

My graduate thesis has aimed to address the questions on why target proteins of TNKS have short half-lives and how they are degraded rapidly. I am interested in understanding the mechanism that allows for very fast and efficient degradation of labile proteins. I have hypothesized that PTM of proteins by ADP-ribosylation provides a molecular scaffold to assemble them together with a specific E3 ubiquitin ligase and proteasomes, thereby locally concentrating all “players” and causing accelerated degradation of target proteins (Fig 1.6).

I have proposed that TNKS forms a complex with Iduna, which is inactive when bound to non-PARylated TNKS in the cell. After substrate binding to TNKS and subsequent PARylation, Iduna binds an internal unit of PAR. This binding causes a conformational change in the RING domain of Iduna, activating its ubiquitin ligase activity and enabling the poly-ubiquitylation of the substrate. As a consequence, TNKS may be promoted for its PARsylation activity to assemble PARsylation scaffold. Finally, I suggest that PI31 may play a role in proteasome recruitment. 26S proteasomes, therefore, can be recruited to the scaffold and concentrating them in the local environment for the rapid breakdown of the target proteins (Fig 1.6).

The overarching goal of this thesis is to understand the mechanisms that allow for rapid and efficient protein degradation. I combined the power of fly genetics with that of molecular
and cellular tools to investigate the regulatory functions of PARsylation and Iduna on TNKS-coupled protein degradation. I, therefore, i) determined the physiological role(s) of Iduna by generating loss-of-function mutants, ii) understood the role of Iduna for TNKS-coupled PARsylation and proteolysis, iii) investigated whether ADP-ribosylation served as a molecular scaffold to accelerate the proteolysis of TNKS target proteins by mapping ADP-ribose acceptor site(s) of TNKS and its possible substrates, and finally iv) identified the possible regulatory proteins that have an impact on PARsylation-mediated protein breakdown.

This thesis addresses the role of PARsylation in TNKS-mediated rapid protein degradation. This work, therefore, provides novel mechanistic insights into the regulation of protein quality control. Ultimately, these results may also provide a conceptual basis to guide the development of new therapies.
Figure 1.9 Hypothetical working model. ADP-ribosylation provides a molecular scaffold to assemble them together with the specific E3 ubiquitin ligase and proteasomes, thereby locally concentrating all “players” and causing accelerated degradation of target proteins. We propose that TNKS forms a complex with Iduna, which is inactive when bound to non-PARylated TNKS in the cell. After substrate binding to TNKS and subsequent PARylation, Iduna binds an internal unit of PAR. This binding causes a conformational change in the RING domain of Iduna, which activates its ubiquitin ligase activity, enabling the poly-ubiquitinylation of the substrate. As a consequence, TNKS may be promoted for its ribosylation activity to assemble poly-ADP-ribose scaffold. Finally, we suggest that PI31 may have a role in proteasome recruitment. 26S proteasomes, therefore, can be recruited to the scaffold and concentrated in a local environment for rapid break down of the target protein.
2 Axin proteolysis by Iduna is required for the regulation of stem cell proliferation and intestinal homeostasis in *Drosophila*

2.1 Summary

The self-renewal of intestinal stem cell is controlled by Wingless/Wnt-β catenin signaling in both *Drosophila* and mammals. Since Axin is a rate-limiting factor in Wingless signaling its regulation is essential. Iduna is an evolutionarily conserved ubiquitin E3 ligase that has been identified as a critical regulator for degradation of ADP-ribosylated Axin and thus of Wnt/β-catenin signaling. However, its physiological significance remains to be demonstrated. Here, I generated loss-of-function mutants of *Iduna* to investigate its physiological role in *Drosophila*. I show genetic depletion of *Iduna* causes the accumulation of both TNKS and Axin. Increase of Axin protein in enterocytes non-autonomously enhanced stem cell divisions in the *Drosophila* midgut. Enterocytes secreted Unpaired and thereby stimulated the activity of the JAK-STAT pathway in intestinal stem cells. A decrease in *Axin* gene expression suppressed both the over-proliferation of stem cells and restored their numbers to normal levels in *Iduna* mutants. These findings suggest that Iduna-mediated regulation of Axin proteolysis is essential to maintain tissue homeostasis in the *Drosophila* midgut.
2.2 Introduction

The evolutionarily conserved Wnt/β-catenin signaling pathway is a main regulator of animal development. It controls proliferation, differentiation and regeneration of adult tissues (Herr et al., 2012; Nusse and Clevers, 2017). The Wingless pathway is also involved in adult tissue self-renewal in Drosophila (Lin et al., 2008). Genetic depletion of proteins in the Wingless pathway, such as Tcf, arr, dsh and pygo, leads to inhibition of Wingless signaling activation which in turn causes over-proliferation of stem cells in the Drosophila midgut (Kramps et al., 2002; Wang et al., 2016a, b; Tian et al., 2016). However, inactivation of Wnt signaling in the mouse small intestine decreases the proliferative potential of stem cells (Fevr et al., 2007; Korinek et al., 1998). On the other hand, mutations resulting in the over-activation of the Wnt/β-catenin pathway promote tumorigenesis (Clevers and Nusse, 2012; Andreu et al., 2005; Korinek et al., 1997 and 1998; Morin et al., 1997). For instance, mutations in the adenomatous polyposis coli (APC) gene cause a hereditary colorectal cancer syndrome called familial adenomatous polyposis (Kinzler et al., 1991; Nishisho et al., 1991). Axin loss-of-function mutations are found in hepatocellular carcinomas, while oncogenic β-catenin mutations are described in colon cancer and melanoma (Rubinfeld et al., 1997). Consequently, intense efforts have been made to target this pathway for therapeutic purposes (Clevers and Nusse, 2012).

A key feature of the Wnt/β-catenin pathway is the regulated proteolysis of the downstream effector β-catenin by the β-catenin degradation complex. The principal components of this complex are APC, Axin and Glycogen synthase kinase 3β (GSK3β) (Kramps et al., 2002; Hamada et al., 1999; Salic et al., 2000; Lee et al., 2003). Axin, a critical scaffold protein in the β-catenin degradation complex, is the rate-limiting factor of Wnt signaling and its protein levels are regulated by the Ubiquitin-Proteasome System (UPS) (Li et al., 2012). Axin is targeted for
degradation by the combined action of the TNKS and the ubiquitin E3-ligase Iduna/Ring finger protein 146 (RNF146) (Zhang et al., 2011). Both genetic and pharmacological studies suggest that UPS-dependent degradation of Axin occurs in a specific temporal order. Iduna initially exists in an inactive state but binding to its iso- or PARsylated targets causes allosteric activation of the enzyme (DaRosa et al., 2014). In the first step, TNKS binds to Axin and ADP-ribosylates Axin using NAD+. Then, Iduna recognizes and binds to ADP-ribosylated Axin via its WWE domain and poly-ubiquitylates Axin. Following the ADP-ribosylation and ubiquitination, post-translationally modified Axin is rapidly degraded by the proteasome (DaRosa et al., 2014; Wang et al., 2016 a, b; Croy et al., 2016; Callow et al., 2011). This tight control suggests a crucial function for Iduna to regulate the Wnt-β catenin pathway.

Because the stability of Axin is partially regulated by TNKS-mediated ADP ribosylation, specific small-molecule inhibitors have been developed to inhibit Wnt-signaling (Lu et al., 2009; Huang et al., 2009). For example, XAV939 targets the ADP-ribose polymerase activity of TNKS and increases Axin levels, which in turn destabilizes β-catenin to inhibit Wnt signaling (Huang et al., 2009). There are two TNKS isoforms in mammalian cells (Hsiao et al., 2006). Tnks1 knockout and Tnks2 knockout mice are overall normal; however, double knockout of Tnks1/2 causes early embryonic lethality, which indicates their redundancy in mouse development (Hsiao et al., 2006; Chiang et al., 2008). On the other hand, inactivation of the single Drosophila Tnks gene produces viable flies that have slightly increased Axin levels and abnormal proliferation of intestinal stem cells, but otherwise display no overt defects (Wang et al., 2016 a, b; Feng et al., 2014; Yang et al., 2016; Tian et al., 2016). The exact physiological function of Iduna, however, remains to be determined. In order to address this question, I generated and characterized Drosophila Iduna
loss-of-function mutants and demonstrate a critical function of this pathway for stem cells in the *Drosophila* intestinal tract.

The *Drosophila* genomes encode four isoforms of CG8786/Iduna/RNF146, which is evolutionarily conserved from *Drosophila* to human. In this study, I concentrated on the physiological function of Iduna in the adult *Drosophila* midgut, which shares several striking similarities with the mammalian small intestine but offers greater anatomical and genetic accessibility (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Markstein et al., 2014). Under normal conditions, Wingless signaling controls stem cell proliferation and cell fate specification in adult midgut (Tian et al., 2016). Here, I showed that Iduna has a physiological function to regulate the proteolysis of both TNKS and Axin. Inactivation of *Iduna* results in increased numbers of midgut stem cells and progenitors due to over-proliferation. I found that Axin accumulation in enterocytes promotes the secretion of Unpaired, a cytokine that binds to the Domeless receptor and activates the JAK-STAT pathway in stem cells and thereby promotes stem cell division. Significantly, reducing *Axin* expression by half restores the numbers of ISC. These findings indicate that regulation of Axin proteolysis by Iduna is necessary to control intestinal homeostasis in *Drosophila*, and it provides physiological evidence for the idea that the functions of TNKS and Iduna are tightly coupled.
2.3 Results

2.3.1 Iduna plays a role in Axin degradation

To examine the *in vivo* function of *Drosophila* Iduna, CRISPR-Cas9 genome editing was used to generate *Iduna* mutants. *Iduna* is located on the 3rd chromosome of *Drosophila*. I designed a specific guide RNA that targets *Iduna*’s first exon and identified two mutant alleles by Sanger sequencing: *Iduna*\(^{17}\) and *Iduna*\(^{78}\), which have 4-nucleotides and 2-nucleotides deletions, respectively (Fig 2.1A-B). These deletions are close to the translation start side of *Iduna*.

Next, I assessed the levels of mRNA and protein expression in these mutants. Using reverse transcript PCR analyses, I found significantly reduced amounts of *Iduna* transcripts in the *Iduna*\(^{78}\) mutant. On the other hand, I was unable to detect any *Iduna B* and *C/G* transcripts in the *Iduna*\(^{17}\) allele (Fig 2.1C). Moreover, no Iduna protein was detected in either of these mutants, indicating that they represent null-mutations (Fig 2.1D). Finally, genetic analyses of these alleles in trans to a larger deletion indicate that both alleles are complete loss-of-function mutations. *Iduna* mutants were crossed to *Drosophila* deficiency lines [Df(3L) Exel6135, Df(3L) ED228)] and also to each other and all combinations were viable as trans-heterozygotes.

I examined the larval development of *Iduna* mutants and Oregon R but did not observe any differences in the numbers of hatched eggs (Fig 2.2A-B), pupated larvae or eclosed adult *Drosophila* (Fig 2.2C) between *Iduna* mutants and wild type. *Iduna*-null adult flies had no overt morphological defects when compared with wild type controls, although, *Iduna* mutants did not live as long as control flies (Fig 2.3A). *Iduna* inactivation, therefore, decreased average lifespan compared to wild type (Fig 2.3B).
Figure 2.1 *Iduna*<sup>17</sup> and *Iduna*<sup>78</sup> are null mutants. A- *Iduna*<sup>17</sup> and *Iduna*<sup>78</sup> have deletions of 4 and 2 nucleotides, respectively, introduced early stop codons and led to truncations of Iduna protein. sgRNA against Iduna was designed to generate small nucleotide deletions, close to its translation initiation site. The three nucleotides were highlighted with red to indicate the location of Cas9 cleavage site. B- *Iduna* loss-of-function mutants, *Iduna*<sup>17</sup> and *Iduna*<sup>78</sup>, were isolated by Sanger sequencing. C- *Iduna*<sup>17</sup> had no detectable *Iduna* transcripts, and *Iduna*<sup>78</sup> had severely reduced *Iduna* mRNA based on RT-PCR. 7-day-old adult females were analyzed for expressions of *Iduna*, *Ribosomal protein 49* (a house keeping gene) and *Ornitate aminotransferase*. D- *Iduna*<sup>17</sup> and *Iduna*<sup>78</sup> have no detectable protein. Endogenous Iduna protein was detected by immunoblotting. Anti-Iduna antibody was generated in guinea pigs and 20μg total protein lysates of 7-day-old adult females were analyzed by immunoblotting. β-actin was used as a loading control. Both *Iduna* alleles have no detectable protein and behave genetically as null-alleles.
Figure 2.1

A

target sequence
ATGTGCAACAGCGCTCCACAGGAGGCAGATTCGAG

Chr 3L
Iduna genomic locus

WT
ATGTGCAACAGGCCTCCACAGGAGGCAGATTCGAG

Mutant#17
ATGTGCAACAGGCCTCCACAGGAGGCAGATTCGAG

Mutant#78
ATGTGCAACAGGCCTCCACAGGAGGCAGATTCGAG

Sequence
Deletion size(nt)

WT
ATGTGCAACAGGCCTCCACAGGAGGCAGATTCGAG

Mutant#17
ATGTGCAACAGGCCTCCACAGGAGGCAGATTCGAG (-4)

Mutant#78
ATGTGCAACAGGCCTCCACAGGAGGCAGATTCGAG (-2)

B

Wt

C

bp

Iduna B

1650
1000
850

2000
1650

1000

200

1000

300

200

Oat

C/G

Iduna

D

kDa

Iduna

(short exposure)

WB: α-dIduna

Iduna

(long exposure)

β-actin

Wt

Wt

17/78

17/Def

100

75

50

37
Figure 2.2 *Iduna* inactivation does not cause developmental defects in *Drosophila*. **A**- *Iduna* mutants (*Iduna<sup>-/-</sup>: *Iduna*<sup>17/17</sup>) did not have defects in hatching their eggs. n>250 for each genotype. **B**- *Iduna* transheterozygous (*Iduna<sup>-/-</sup>: *Iduna*<sup>17/78</sup>) mutants did not have defects in hatching their embryos. n>100 for each genotype. **C**- *Iduna* mutant larvae could be pupated and eclosed to adult *Drosophila*. n>100 for each genotype. Oregon R flies used as wild type flies. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. ‘`ns`’ means ‘`not significant``."
Figure 2.3 Loss-of-function mutants of *Iduna* display increased mortality under reduced nutrient conditions. A- *Iduna* \(^{17/17}\) mutant flies lived slightly shorter than wild type flies. B- *Iduna* inactivation decreased average lifespan compared to wild type flies. Flies were aged matched at 24-25\(^{\circ}\)C. n>100 from each genotype. C- *Iduna* \(^{17/78}\) flies were analyzed on 5% sucrose diet. For statistical analyses, I used the Mantel-Cox and Gehan-Breslow-Wilcoxon tests to compare survival curves between *Iduna* mutants and Oregon R wild type flies. 2-day-old mutant or wild type female flies were collected and kept on 5% sucrose diet at 28\(^{\circ}\)C. n=100 from each genotype.
However, they displayed increased mortality upon nutrient deprivation. I challenged two-day-old mutant and wild type adult females with a 5% sucrose diet at 28°C. Mutant flies died within 17 days, while 70-80% of wild type flies were still viable at this time (Fig 2.3C).

Iduna is one of the key components of the machinery that degrades Axin whose ADP-ribosylation by TNKS is crucial for mammalian Wnt-β catenin signaling (Li et al., 2012). I detected increased levels of endogenous Axin in the lysates from Iduna mutant midguts compared to controls (Fig 2.4A). Mammalian Iduna recognizes both ADP-ribosylated (ADPR) TNKS and Axin via the R163 residue in its WWE domain (Zhang et al., 2011). The R163 residue is conserved in evolution and corresponds to R252 in the Drosophila WWE domain (Fig 2.4B). To examine the level of endogenous ADPR-Axin in Iduna mutants, ADPR-Axin was pulled down with wild type-WWE or R252A-WWE-mutant recombinant proteins (Fig 2.4C). This analysis revealed that Iduna mutants had a more than two-fold increase in ADPR-Axin in their midguts compared to the wild type (Fig 2.4D-E). These results suggest that Iduna promotes Axin degradation in vivo. To further understand the contribution of Iduna inactivation for both TNKS and Axin proteolysis in Drosophila, UAS-Flag-Tnks and UAS-GFP-Axin transgenes were mis-expressed under an eye-specific driver, GMR, in an Iduna mutant background (Fig 2.5A). To detect mis-expressed GFP-Axin and Flag-TNKS levels, total proteins were extracted from 5-day-old male heads and analyzed by immunoblotting (Fig 2.5C and E). I found that Iduna mutants had 2.5-fold more mis-expressed GFP-Axin protein when compared to the control (Fig 2.5D). These mutants also had 3.5-fold more ectopic expressed Flag-tagged TNKS as well (Fig 2.5F).
Figure 2.4 *Iduna* inactivation leads to increased Axin protein levels in the midgut. **A**- *Iduna* mutant midguts have elevated levels of Axin protein compared with controls. Midguts of 7-day-old adult females were dissected, lysed and analyzed by Axin immunoblotting. β-Actin was used as a loading control. **B**- Mammalian Iduna recognizes ADP-ribosylated (ADPR) Axin via the R163 residue in its WWE domain. The R163 residue is conserved in evolution and corresponds to R252 in the Drosophila WWE domain. *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Dm*, *Drosophila melanogaster*. **C**- Recombinant wild type and R252A mutants were used as biochemical sensors to pull down the ADPR-Axin from Drosophila midguts. Myc-tagged WWE proteins were expressed and purified from Drosophila S2R+ cells by immunoprecipitation. **D**- Inactivation of *Iduna* leads to accumulation of ADPR-Axin. Wild-type Myc-tagged-WWE protein pulled down ADPR-Axin. In contrast, the R252A mutant did not interact with modified Axin. Following immunoprecipitation (IP), eluted proteins were analyzed with an anti-PAR antibody. The 50 kDa heavy chain IgG is indicated on the blot. **E**- *Iduna* inactivation results in 2.3-fold more ADPR-Axin protein in the midgut. Western blot quantification of two independent experimental replicates; ADPR-Axin levels were normalized to the control lines. Flies were fed with regular diet at 24-25°C. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. *p*<0.001 is indicated as *** and *p*<0.0001 was marked as ****.
Figure 2.4

A. Iduna

B. WWE domain RNF146

Hs. GRRRKIKR

Mm. GRRRKIKR

Dm. TRCRVKR

252

C. Iduna17/78 or Iduna17/+ flies

midgut dissection

midgut total protein extraction

Recombinant Myc-tagged WWE-R252 or WWE-R252A

WWE-pull down

WWE-R252

WWE-R252A

D. IP: Myc-Wt-WWE

IP: Myc-R252A-WWE

17/78

17/1+

17/1+

17/78

17/78

100

75

50

20

10

ADPR- dAxin

* WB: α-Axin (le)

ADPR- dAxin

* WB: α-Axin (le)

Myc-WWE

* Heavy chain IgG

E. Relative ADPR-Axin level

Wt-WWE

R252A-WWE

Iduna17/78

Iduna17/1+

***

****
Figure 2.5 *Iduna* depletion leads to increased Axin and TNKS protein levels in the *Drosophila* eye. A- Mis-expression of GFP-Axin in 5-day-old adult male eyes did not result in an obvious eye phenotype. B- Mis-expressed TNKS however led to rough eye phenotype in 5-day-old adult males. *Iduna* inactivation promoted the eye phenotype. C- *Iduna* inactivation leads to mis-expressed GFP-Axin elevation. A *UAS-GFP-Axin* reporter transgene was expressed under *GMR* driver in the *Iduna* mutants or wild type. D- *Iduna* mutants had 2.5-fold increased GFP-Axin protein. Quantification of mis-expressed GFP-Axin immunoblotting results are based on two repeats of independent replicates and Axin protein levels were normalized to β-actin. E- Flag-tagged mis-expressed TNKS protein accumulates in *Iduna* mutants. A *UAS-Flag-Tnks* reporter transgene was expressed under the *GMR-Gal4* driver in *Iduna* mutants or wild type. F- *Iduna* mutants had 3.5-fold increased levels of Flag-tagged TNKS. Quantification of Flag-TNKS immunoblotting. 5-day-old adult male heads were dissected and 20µg of total protein lysates were analyzed by immunoblotting to assess the levels of GFP-Axin or Flag-tagged TNKS using α-GFP or α-Flag antibodies, respectively. Western blot quantification was performed based on two independent experimental replicates, and protein levels were normalized with β-actin. Oregon R flies were used as a wild type control. Flies were fed with regular diet at 24-25°C. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. *** *p*<0.001
Figure 2.5

A

GMR Gal4> UAS-GFP-Axin; Iduna^{+/+}

B

GMR Gal4> UAS-GFP-Axin; Iduna^{-/-}

C

GMR-Gal4>UAS-GFP-Axin:

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GFP-Axin

\[ \beta \text{-actin} \]

D

Relative Axin protein level

Wild type

Iduna^{-/-}

E

GMR-Gal4 > UAS-Flag-Tankyrase:

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Flag-Tankyrase

\[ \beta \text{-actin} \]

F

Relative TNKS protein level

Wild type

Iduna^{-/-}
When I examined the eye morphology, GFP-Axin mis-expression did not cause an obvious eye phenotype (Fig 2.5A). In contrast, mis-expressed Flag-tagged TNKS led to a rough eye phenotype, which was more severe when Tnks was mis-expressed in Iduna\(^{-/}\) homozygous mutants compared to Iduna\(^{+/}\) heterozygous animals (Fig 2.5B). Recently, it was also reported that mis-expressed TNKS promotes apoptosis in the Drosophila eye due to the activation of JNK signaling (Feng et al., 2018).

In order to examine whether Axin is a target for Iduna-mediated degradation, I also mis-expressed a UAS-GFP-Axin transgene under the enterocyte (EC) specific temperature sensitive Myo1A-Gal4 driver (Fig 2.6A) and saw 2-2.5-fold more Axin in Iduna mutants compared to controls (Fig 2.6B). To investigate the cellular levels of Myo1A driven GFP-Axin in ECs, I examined FRT80B, Iduna mutant clones and found that mutant EC clones had more GFP-Axin when compared to their neighboring cells (Fig 2.6C). Taken together, these observations suggest that Iduna plays a role in promoting the degradation of both Axin and TNKS.

### 2.3.2 Iduna is required to control the proliferation of intestinal progenitors in the Drosophila midgut

Attenuations in the Wingless pathway affect the proliferation of stem cells in the Drosophila midgut. For instance, inactivation of Tcf, arr, armadillo, dsh, and pygo leads to suppression of Wingless signaling, which in turn causes more stem cell division (Kramps et al., 2002; Wang et al., 2016a and 2016b; Tian et al., 2016). Furthermore, Apc and Tnks mutations cause elevation of Axin, which reduces Wingless signaling which in turn promoting mitosis of stem cells in the Drosophila midgut (Wang et al., 2016 a, b; Tian et al., 2016). Hence, the Wingless signaling pathway is required to control ISC proliferation in Drosophila (Xu et al, 2011; Cordero et al., 2012; Tian et al., 2016).
Since *Iduna* mutants have elevated Axin, I considered that *Iduna* inactivation might cause aberrant proliferation of stem cells in the *Drosophila* midgut. Just as the mammalian intestine (Korinek et al., 1998), the *Drosophila* midgut has intestinal stem cells (ISCs), which give rise to all intestinal compartments (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ISCs specifically give rise to two types of daughter progenitor cells: undifferentiated enteroblast (EBs) and pre-enteroendocrine cells (Pre-EE). EBs and pre-EEs differentiate into ECs and EEs cells, respectively (Ohlstein and Spradling, 2006; Xu et al., 2011) (Fig 2.7A). ISCs can be distinguished from ECs by their cell size and marker proteins (Ohlstein and Spradling 2006; Xu et al., 2011). ISCs are small, express cell membrane-associated Armadillo, and lack nuclear expression of Prospero (Fig 2.7B). In contrast, nuclear Prospero staining is a marker of small-sized differentiated EE cells (Fig 2.7B).

ISCs are also marked by the expression of *escargot* (*esg*), a transcription factor, whose GFP reporter allows tracing of stem and progenitor cells during development (Ohlstein and Spradling, 2006) (Fig 2.7B). Using the *esg>*GFP marker, I first analyzed 9-day-old female flies which were fed with a 5% sucrose diet for seven days at 28°C and saw an approximately twofold increase in the numbers of *esg>*GFP positive ISCs/progenitors in the midgut of *Iduna* mutants compared to controls (Fig 2.8A-B). *Iduna* inactivation increased the numbers of Arm+/Pros− stem cells in midguts (Fig 2.8C) upon nutrient deprivation.
Figure 2.6 *Iduna* inactivation causes increased mis-expressed Axin protein levels in the midgut. **A-** *Iduna* mutant midguts had elevated mis-expressed GFP-Axin protein. Midguts of 7-day-old adult females, which were expressing GFP-Axin under the temperature sensitive *Myo1A-Gal4* driver, were dissected, lysed and analyzed by GFP immunoblotting. *Iduna* mutants had more Axin protein compared to the wild type. 20µg total intestinal lysates were analyzed by GFP immunoblotting and α-tubulin was used as a loading control. **B-** Loss-of-*Iduna* resulted in 2.2-fold GFP-Axin accumulation in the midgut. Western blot quantification was performed based on two independent experimental replicates, and protein levels were normalized to α-Tubulin. **C-** *Iduna* mutant clones have elevated mis-expressed GFP-Axin compared to their WT neighbors. A *UAS-GFP-Axin* transgene was expressed under the temperature sensitive *Myo1A-Gal4* driver in the FRT80B-*Iduna*¹⁷ mutant. Midgut mutant clones were induced during larval development by daily incubation at 37°C for 1h. Adult female FRT80B-Nls-Red/FRT80B-*Iduna*¹⁷ flies were collected after eclosion, incubated at 28-29°C and analyzed on day 7. Unlabeled cells represent *Iduna* mutant clones, whereas cells stained for nuclear RFP are either wild type or *Iduna* heterozygous. *p*<0.001 is indicated as *** and *p*<0.0001 was marked as ****. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10µm
Figure 2.6

(A) Myo1A<sup>ts</sup>→UAS-GFP-Axin:

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<th>kDa</th>
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<th>111&lt;sup&gt;−/−&lt;/sup&gt;</th>
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GFP-Axin

β-actin

(B) Relative Axin protein level

![Bar graph showing relative Axin protein level for Wild type and Iduna<sup>−/−</sup>](image)

Wild type

Iduna<sup>−/−</sup>

***

(C) Myo1A<sup>ts</sup>

FRT80B, Iduna<sup>−/−</sup>

UAS-GFP-Axin

RFP<sup>nls</sup>

Merged

![Images showing fluorescence microscopy of Myo1A<sup>ts</sup> and RFP<sup>nls</sup> combined](image)
To test whether the increased number of ISCs was dependent on nutrient deprivation, I examined midguts of 7-day-old female mutants and controls on regular diet. I saw again an approximately twofold increase in the numbers of both esg>GFP positive (Fig 2.8E-F) and Arm+/Pros- stained (Fig 2.8G-H) stem cells-progenitors under these conditions. Therefore, increased ISC numbers in Iduna mutants are independent of diet.

To exclude the possibility that Iduna mutant flies raised on regular diet had reduced nutrient uptake, I monitored fly feeding by an acid blue 9 colorimetric assay (Mattila et al., 2018). I noticed no decrease in food intake in Iduna mutants kept on regular diet at 24-25°C compared to controls (Fig 2.8D). These results show that Iduna inactivation promotes the numbers of midgut stem cells independent of diet and food intake. Finally, I analyzed the midgut cell composition in Iduna mutant and control flies. I observed a slight increase in the total midgut cell number of Iduna mutants (Fig 2.9A). However, there were no significant differences in the number of EC and EE cells (Fig 2.9B-C). Therefore, Iduna is not required for differentiation of ISCs (Fig 2.9D-E). Collectively, these observations indicate that Iduna inactivation selectively affects ISC numbers.
Figure 2.7 Intestinal stem cells in the *Drosophila* midgut give rise to ECs and EE cells. A- ISCs give rise to two different types of daughter progenitors, undifferentiated EBs and Pre-EEs. EBs and pre-EEs differentiate into ECs and EEs cells, respectively. B- ISCs and ECs can be distinguished by their cell sizes, high level of membrane-associated Armadillo, and lack of nuclear Prospero. On the other hand, differentiated EEs are small and can be identified by nuclear Prospero staining. Finally, small-sized ISCs are stained with Armadillo but not with nuclear Prospero. In the image, small ISCs were co-stained with escargot (green), and Armadillo (red). Small EEs were shown with the red arrow and stained with Armadillo (red) and nuclear Prospero (red). Bigger cells are enterocytes whose cell membrane is stained with Armadillo (red). DAPI staining in blue marks the cell nucleus.
Figure 2.8 *Iduna* mutants have increased numbers of intestinal stem and progenitor cells in their midgut. **A**- Upon nutrient deprivation, there is an approximately twofold increase in the numbers of *esg>GFP* expressing stem cells/progenitors in the midgut of *Iduna* mutants compared to controls. **B**- Quantification of *esg>GFP* positive stem cells and progenitors from adult flies of indicated genotypes. n=6 from each genotype. **C**- *Iduna* inactivation increases the numbers of Arm⁺/Pros⁻ ISCs upon nutrient deprivation. 9-day-old female flies, fed with 5% sucrose diet for seven days at 28°C, were examined in A-B and C. **D**- There is no decrease in food intake in *Iduna* mutant when flies kept under the regular diet. Quantification of adult fly nutrient uptake by a calorimetric assay from regular dietary condition in *Iduna* mutants and Oregon R. **E**- Under normal diet, inactivation of *Iduna* also promotes the proliferation of the *esg*-GFP-labeled intestinal stem and progenitor cells in the *Drosophila* midgut. **F**- Quantification of *esg>GFP*⁺ stem cells and progenitors from adult flies of indicated genotypes. In wild type, 25-30% of posterior midgut cells are stem cells, as assessed by *esg>GFP* expression. In contrast, 55-60% of the total cell population in *Iduna* mutants expressed the stem marker *esg>GFP*, representing a greater than 2-fold increase. **G**- *Iduna* mutants have more Arm⁺/Pros⁻ ISCs in the midgut. **H**- Quantification of Arm⁺/Pros⁻ ISCs from adult flies of indicated genotypes. The midguts of 7-day-old adult females were analyzed by confocal microscope. For the consistency, posterior midgut R5 region was analyzed in this study. *Iduna^{17/+}* flies were used as control. n>12 from each genotype. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10 μm. *p<0.001* was indicated as *** and *p<0.0001* was marked as ****.
Figure 2.8

5% sucrose diet

Iduna^{w1}

Iduna^{1/17}

A

esp-GFP/DAPI

C

Amp/Prol/DAPI

B

esp-GFP* cells upon nutrient deprivation

D

Adult fly nutrient uptake

Regular diet

Control

Iduna^{1/17}

E

Esp-GFP/DAPI

G

Amp/Prol/DAPI

F

esp-GFP* cells/field

H

Amp/Prol (SCs/field)
Figure 2.9 Iduna is not required for differentiation of ISC. **A**- There were more cells in the midgut of *Iduna* mutants. **B-C**- No differences in the numbers of ECs and EEs were observed in *Iduna* mutants. **D**- Iduna is not required for the differentiation of ISCs into ECs. ECs were marked with GFP expressed under the control of EC specific driver, *Myo1A-Gal4*. Control cells are shown with nuclear RFP, and *Iduna* mutant clones are marked with a white line. **E**- Genetic depletion of *Iduna* did not affect the differentiation of ISCs into EEs. Mutant clones in 7-day-old adult midguts were analyzed. Small nuclear Prospero-positive EEs are labeled with yellow arrows in *Iduna* mutant clones. Control cells are labeled with nuclear RFP, and mutant clones are marked with a white line. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10µm
Figure 2.9
The observed increase in stem cell numbers could be the result of aberrant stem cell proliferation or inhibition of their differentiation. To distinguish between these possibilities, I first assessed cell proliferation by dissecting 7-day-old mutant and wild type females. Following an hour EdU-labeling of the dissected midguts, I observed that Iduna mutants had more EdU-positive cells (Fig 2.10A-C). Moreover, phospho-Ser-Histone H3 (pH3) immunostaining (Fig 2.10D-E) also revealed a significant increase in pH3\(^+\) mitotic cells in the midgut of 7-day-old female Iduna mutants (Fig 2.10D-F). These findings suggest that stem cells undergo increased proliferation in the midgut of Iduna mutants. To address whether there was an inhibition of differentiation in Iduna mutants, I generated FRT80B, Iduna mutant clones (Theodosioiu and Xu, 1998). I found that ECs and EEs were present in the 5-day-old female mutant clones, demonstrating that Iduna was not essential for differentiation of ISCs into daughter cells (Fig 2.9D-E).

### 2.3.3 Regulation of Axin proteolysis by Iduna is necessary for normal ISC proliferation

One possible mechanism by which Iduna may control the proliferation of ISCs in the Drosophila midgut is through modulating the concentration of Axin. To determine whether a reduction of the elevated Axin levels could reduce ISC numbers in Iduna mutants, they were recombined with Axin mutants and then back-crossed with Iduna mutants to generate flies that were homozygous mutant for Iduna\(^{-}\) and heterozygous for Axin\(^{+/\cdot}\). Strikingly, a reduction of the Axin gene dosage by 50% restored ISC numbers to wild type levels in Iduna mutants (Fig 2.11A). Compared to 7-day-old female controls, Iduna mutants had an approximately twofold increase in the number of Arm\(^{+/\cdot}\)/Pros\(^{-}\) as well as pH3\(^+\) mitotic stem cells (Fig 2.11B-C).
Figure 2.10 *Iduna* inactivation enhances proliferation of intestinal stem cells. **A-B**- Genetic depletion of *Iduna* leads to over-proliferation of intestinal stem cells in the midgut. EdU was used as a proliferation marker in 7-day-old mutant or wild type female flies. Following fixation, EdU incorporation was analyzed by confocal microscope. **C**- Increased numbers of EdU+ stem cells were seen in *Iduna* mutants, indicating increased cell proliferation. Posterior midguts were analyzed for quantification. **D-E**- *Iduna* mutants display elevated phospho-Ser-Histone 3 (pH3)-positive ISCs. **F**- Iduna inactivation leads to an increase of pH3+ mitotic stem cells in the midgut. Quantification of pH3+ proliferating cells was done in the whole midgut. 7-day-old mutant or wild type female flies were examined. Flies were fed with regular diet at 24-25°C. n>12 from each genotype. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10µm. *p*<0.0001 was marked as ****.
Figure 2.10

A and B: Wild type and Iduna^{17/18} comparison of EdU+/DAPI staining.

C: Box plot showing EdU+ISCs/field.

D and E: Wild type and Iduna^{17/18} comparison of Arm/Pros/pH3/DAPI staining.

F: Box plot showing pH3+ISCs/midgut.
On the other hand, reducing the *Axin* gene dosage by 50% in an *Iduna*-null background yielded numbers of ISCs and the pH3+ stem cells comparable to 7-day-old wild type females. These results suggest that small changes in the levels of Axin have profound effects on stem cell number, and that regulation of Axin degradation by Iduna is necessary for normal ISC proliferation.

I observed that *Iduna* mutants had 2-fold more Axin in the *Drosophila* midgut. This indicates that defects in Axin degradation might cause over-proliferation of stem cells due to inhibition of Wingless signaling. Therefore, I analyzed a reporter for the Wingless pathway target gene, *frizzled-3* (*fz3*). It was previously reported that *fz3*-RFP reporter activity is high at the major boundaries between compartments (Buchon et al., 2013; Tian et al., 2016; Wang et al., 2016 a, b). *fz3*-RFP was strongly expressed in enterocytes at three distinct sites of the midgut: around R1a, R2c, and R5 (Buchon et al., 2013). Therefore, enterocytes are the primary sites of the Wingless pathway activation during intestinal homeostasis (Tian et al., 2016). I analyzed 3-day-old *fz3-Gal4>Gal4* females and consistently observed that *fz3>GFP* was expressed in gradients in the foregut, posterior midgut, as well as the border between the posterior midgut and hindgut (Fig 2.11D). Here, I focused on the posterior midgut-hindgut border to investigate the effect of Iduna on Wingless signaling. Upon *fz3-Gal4* driven RNAi-mediated *Iduna* depletion, I found that *fz3>GFP* activity decreased significantly (Fig 2.11E). I concluded that Iduna stimulates Wingless activity in the posterior midgut by promoting degradation of Axin.

The proliferation of stem cells in the *Drosophila* midgut is regulated by intrinsic signals and also interactions with neighboring cells (Zhou et al., 2013; Tian et al., 2016).
Figure 2.11 A 50% reduction of *Axin* restores ISC numbers in the *Drosophila* midgut. A- Reducing the *Axin* gene dosage by half restores the number of Arm+/Pros- ISCs. *Axin*044230 is a complete *Axin*-null mutant, and *Axin*E77 is a loss-of-function truncation allele (Q406X). Midguts of 7-day-old adult females of the indicated genotypes were dissected and analyzed by confocal microscope following Armadillo, Prospero and DAPI staining. *Axin*+/-, *Iduna*17/+ served as control. Reducing the Axin gene dosage by 50% in *Iduna*17/17 mutants decreased the number of ISCs to normal levels. B- Quantification of ISC numbers. Reducing the *Axin* gene dosage by half fully suppressed the increased numbers of Arm+/Pros- ISCs in *Iduna*17/17 null-mutants. C- Reducing *Axin* gene expression suppressed the proliferation of ISCs in the *Iduna*17/17 null-mutant. p<0.0001 was marked as ****. D- *frizzled 3* (fz3) is a Wingless target gene and a GFP-reporter construct was used here to visualize Wg-activity in the midgut (Buchon et al., 2013; Tian et al., 2016; Wang et al., 2016 a, b). In wild type, fz3>GFP is highly expressed in a graded fashion in the foregut (f), the posterior midgut (p) as well as the posterior midgut-hindgut border, but not in the anterior midgut (a) or the hindgut proper (h). Right-hand image is a higher magnification image of the fz3>GFP near the midgut-hindgut boundary (Scale bar: 20µm). 7-day-old female midguts were analyzed. E- *fz3*-Gal4 driven *Iduna* depletion inhibits Wingless activity. RNAi-mediated down-regulation of *Iduna* led to significant reduction of fz3>GFP; white-RNAi served as a control. The arrow indicates the border between the posterior midgut and the hindgut (b). 3-day-old female midguts were analyzed. Flies were fed with regular diet at 24-25°C. One-way ANOVA was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10µm.
Figure 2.11

A: Control, Iduna^{17/17}, Axin^{677/+}, Iduna^{17/17}, Axin^{G044230-}, Iduna^{17/17}

B: Graph showing Arm lin/Pros ISC3s/field.

C: Graph showing pH3^+ ISC3s/midgut.

D: Drosophila midgut and posterior midgut with Drosophila Gal4>GFP/DAPI.

E: White RNAi, Iduna RNAi with fz3 Gal4>GFP.
**Figure 2.12** *Iduna* depletion in ECs leads to over-proliferation of ISCs. A- Over-proliferation of ISCs in *Iduna* mutants is non-cell autonomous. RNAi-mediated *Iduna* knock-down was carried out in the ECs and ISCs/EBs using *Myo1A-Gal4* and *esg-Gal4* drivers, respectively. B- Knock-down of *Iduna* in ECs using the *Myo1A-Gal4* driver led to over-proliferation of Arm+/Pros− ISCs. In contrast, no changes in ISC proliferation were observed upon down-regulation of *Iduna* in ISCs using *esg-Gal4*-driven *Iduna* RNAi. *Myo1A-Gal4>GFP* served as a control. C- EC-specific knock-down of *Iduna* increased the number of pH3+ progenitors. D- Ectopic expression of *Iduna* in ECs inhibits over-proliferation of ISCs. A UAS-Myc-tagged *Iduna* C/G transgene was generated to perform rescue experiments. E- F- Expression of the UAS-Myc-*Iduna* C/G transgene with the *Myo1A-Gal4* driver resulted in a reduction of the numbers of pH3+ (E-) and Arm+/Pros− (F-) ISCs in the midgut of *Iduna* mutants. Flies were fed with regular diet at 24-25°C. 7-day-old female midguts were analyzed for ISC and mitotic markers. One-way ANOVA was used for statistical analyses. *p*<0.0001 was marked as ****. Error bars represented as means ± s.d. Scale bars: 10µm.
Figure 2.12

A

Myo1A>UAS-GFP

Myo1A>UAS-GFP; Iduna RNAi

Myo1A>UAS-GFP; Iduna RNAi

B

Arm Pros ISCs/field

C

pH3 ISCs/midgut

D

Myo1A>UAS-GFP

Myo1A>UAS-GFP; Iduna17177

Myo1A>UAS-Myc Iduna; Iduna17177

E

F

Myo1A Gal4> UAS-GFP;

Myo1A Gal4> UAS-GFP;
To further investigate whether the observed effects could reflect a cell-autonomous requirement of Iduna in ISCs, or alternatively a requirement in other cells of the midgut, Iduna was specifically targeted in ECs as well as midgut stem and progenitor cells by using the Myo1A-Gal4 and esg-Gal4 drivers, respectively (Fig 2.12A-B). I examined 7-day-old females expressing Iduna-RNAi under the Myo1A or esg drivers. RNAi-mediated knock-down of Iduna in ECs caused a significant increase in Arm+/Pro− stem cell numbers (Fig 2.12B). However, stem cell/progenitor cell-specific knock-down of Iduna did not affect either the stem cell numbers or mitosis in the midgut (Fig 2.12B-C). This suggests that Iduna inactivation causes stem cell over-proliferation by a non-cell-autonomous mechanism, and that perhaps ECs are responsible for stem cell over-proliferation in Iduna mutants. To further test this idea, I ectopically expressed Iduna in ECs and investigated if this could suppress stem cell proliferation in Iduna mutants (Fig 2.12D). Indeed, consistent with this model, I saw that Myo1A-Gal4 driven UAS-Iduna was able to restore normal numbers of stem cells and progenitors (Fig 2.12E-F). Taken together, my results indicate that Iduna plays a physiological role to regulate Wingless signaling in ECs, which is critical for proper ISC proliferation.

I found that Iduna mutants have increased mortality upon nutrient deprivation (Fig. 1C). Following 7 days on a 5% sucrose diet at 28°C, Iduna mutants had more esg>GFP positive cells in the midgut (Fig 2.8A-C). Therefore, I considered that under reduced nutrient diet, hyper-proliferation of ISCs might be responsible for elevated mortality. To test this idea, I first inactivated Iduna in enterocytes by expression of three different RNAi lines under the Myo1A driver.
Figure 2.13 RNAi-mediated Iduna depletion in ECs does not cause mortality upon nutrient deprivation. A- Three different Iduna RNAi lines were expressed under the Myo1A driver. white-RNAi was used as a control. B- There was no significant change on the mean lifespan between white and Iduna-RNAi expressing flies. n=60 for each genotype. C- Iduna-RNAi in EBs also did not result in increased lethality upon 5% sucrose diet at 28°C. n=70 for each genotype. D- Ectopic expression of Iduna under Myo1A driver in ECs does not rescue elevated mortality of Iduna mutants under reduced nutrient diet. 2-day-old mutant or wild type female flies were collected and kept on 5% sucrose diet at 28°C for the experiments in A-D. E- EC-specific ectopic expression of Iduna rescues the hyper-proliferation of midgut stem cells upon nutrient deprivation. 2-day-old females were collected at 24-25°C and their regular diet was replaced with 5% sucrose diet at 28°C. 9-day-old female flies were examined with α-Armadillo and Prospero antibodies. One-way ANOVA was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10µm. For statistical analyses of sucrose diet, I used the Mantel-Cox and Gehan-Breslow-Wilcoxon tests to compare survival curves between Iduna mutant and control flies. Scale bars: 10µm.
**Figure 2.13**

A. RNAi-mediated lduna depletion in enterocytes

B. 5% Sucrose diet mean lifespan (days)

C. Su(H)>White RNAi

D. Ectopic expression of lduna in enterocytes

E. 5% sucrose diet

Myo1A>RFP; lduna^17/+  Myo1A>RFP; lduna^17/17  Myo1A>Iduna; lduna^17/17

Armadillo/Prospero/DAPI
I showed that RNAi-mediated *Iduna* depletion did not increase lethality compared with white-RNAi (Fig 2.13A). There was also no significant change on the mean lifespan between white and *Iduna*-RNAi expressing flies (Fig 2.13B). I also tested EB specific *Iduna* depletion and again found no significant effects on longevity upon nutrient deprivation (Fig 2.13C).

Finally, I expressed UAS-*Iduna* transgene under *Myo1A* driver in ECs to rescue the elevated mortality in the mutants. Whereas the *Iduna* transgene rescued the hyper-proliferation phenotype (Fig 2.13E), it failed to rescue the mortality of mutants on 5% sucrose diet (Fig 2.13D). These findings suggest that Iduna mortality is not caused by dysregulation of midgut stem cell proliferation and point to another role of Iduna in promoting survival under stress conditions.

The fly midgut provides essential physiological functions of the living organism including food digestion and nutrient absorption. It is also a key organ for lipid uptake and distribution. Under normal feeding conditions, lipids digested from dietary food are absorbed by ECs, resynthesized into triglyceride (TG), and packaged into lipoprotein particles that are transported to peripheral tissues for energy supply or storage in a specialized tissue, known as the fat body (Song et al., 2014). Upon starvation, flies consume their lipid storages. Since *Iduna* mutants displayed increased mortality on the reduced nutrient diet, I examined adult fat bodies to test if there might be a lipid deposition defect in *Iduna* mutants. 7-day-old adult females were starved for 12h and 24h. Stored lipids were visualized in the abdominal fat bodies of starved flies by Nile Red staining. Remarkably, stored lipid levels in abdominal fat body cells were visibly reduced upon starvation in *Iduna* mutants (Fig 2.14). I found that *Iduna* mutants constantly lost their fat storages during 24h starvation. Although wild type flies also reduced their fat bodies in 12h, they
sustained their fat reservoirs in 24h (Fig 2.14). My results suggest that Iduna may also have essential role(s) in energy metabolism.

2.3.4 Depletion of Iduna promotes stem cell proliferation through the JAK-STAT pathway

In order to further investigate the mechanism by which Iduna affected ISC proliferation, I explored the function of additional signaling pathways implicated in this system. Because the JAK-STAT pathway has a well-known role in stem cell proliferation, I looked for possible effects of Iduna mutants here (Zeidler et al., 2000; Zoranovic et al., 2013; Zhou et al., 2013; Markstein et al., 2014). I analyzed the JAK-STAT pathway via a 10xStat-GFP reporter in the midgut (Bach et al., 2007). Under regular physiological conditions, Stat-GFP reporter expression was mainly seen in small sized cell populations in the midgut that appear to represent ISCs for several reasons (Fig 2.15). First, Prospero-positive EEs were negative for Stat-GFP (Fig 2.15A). Second, ECs stained with Armadillo were also not expressing the Stat-GFP reporter. Finally, Delta-lacZ positive but Prospero-negative cells for the most part expressed Stat-GFP. However, a minor population of small sized cells was GFP positive but Delta-lacZ negative (white arrows, Fig 2.15B). These cells appeared to be undifferentiated progenitors, such as EBs. 7-day-old Iduna mutants had more Stat-GFP positive cells when compared to controls (Fig 2.15A-F, Fig 2.17A-B). I also generated FRT80B, Iduna midgut mutant clones and observed that these clones had elevated JAK-STAT signaling (Fig 2.16A-B).
Figure 2.14 *Iduna* inactivation promotes fat body wasting upon starvation. 7-day-old adult females were starved for 12h and 24h. Abdominal fat bodies were dissected and stained with Nile Red. Compared to control flies, *Iduna* mutants had severe fat lost during starvation. Oregon R was used as wild type flies.
To confirm elevated JAK-STAT signaling in *Iduna* mutant stem cells, I stained midguts from 7-day-old females for Delta, a previously identified target gene of the JAK-STAT pathway (Jiang et al., 2009). I found that there was indeed more Delta protein in *Iduna* mutants, consistent with elevated JAK-STAT activity (Fig 2.16F).

To test whether activation of JAK-STAT signaling was responsible for aberrant ISC proliferation, I knocked down *Stat92E*, a transcription factor in the JAK-STAT pathway, in ECs as well as in ISCs and EBs. I did not detect dramatic changes in the numbers of mitotic cells when *Stat92E* transcription factor was depleted in ECs (Fig 2.17C). Interestingly, knock-down of *Stat92E* in midgut stem and progenitor cells was sufficient to suppress their increased cell division (Fig 2.17C).

My observations raise the question of how ECs signal ISC proliferation. One possibility is that ECs secrete a factor that activates the JAK-STAT pathway in neighboring ISCs. The JAK-STAT pathway can be activated by cytokines, such as *unpaired* (UPD, UPD2, UPD3) in the *Drosophila* midgut (Ghiglione et al., 2002; Zhou et al., 2013). Since UPD3 is produced in differentiated ECs and in differentiating EBs, I explored the possibility that UPD cytokines could mediate ISC over-proliferation in *Iduna* mutants. For this purpose, I first inactivated *Iduna* with the *upd3-Gal4* driver and found that RNAi-mediated knock-down of *Iduna* resulted in a significant increase of *upd3>GFP* reporter expression in the midgut (Fig 2.17E, Fig 2.18A). *upd3>GFP*-positive cells were mainly ECs, and not EEs or ISCs (Fig 2.17E, Fig 2.18B-C). I then knocked down *Iduna* in ECs and performed a q-PCR to test whether *Iduna* depletion in ECs induced unpaired expression. I detected that EC-specific *Iduna* inactivation resulted in elevated *upd3* gene expression compared to white-RNAi (Fig 2.17D). To suppress the over-proliferation of ISC in *Iduna* mutants, I finally reduced *upd2* and *upd3* gene dosages. Strikingly, I found that
heterozygosity in Δ upd2-upd3 fully suppressed ISC proliferation in Iduna mutants (Fig. 2.17F).
Secreted UPD proteins bind to the Domeless receptor on ISCs (Ghiglione et al., 2002).
Therefore, I tested whether decreasing Domeless levels could also suppress stem cell over-
proliferation in Iduna mutants. Again, this prediction was experimentally confirmed (Fig 2.17F).

Collectively, these observations suggest that Iduna inactivation causes a decrease in
Wingless signaling in ECs which in turn causes elevated JAK-STAT signaling in ISCs, thereby,
resulting in their over-proliferation. I concluded that inactivation of Iduna causes decrease in
Wingless signaling in ECs, which in turn leads to increased secretion of UPD2-3 from these cells
to stimulate over-proliferation of ISCs through the JAK-STAT pathway (Fig 2.17G).
Figure 2.15 Under regular physiological conditions, the Stat-GFP reporter is mainly expressed in midgut stem cells and EBs. A- A 10xStat-GFP reporter was mainly positive in small sized- cells but Prospero-stained EEs were negative for Stat-GFP in the midguts. B- Delta-lacZ positive but Prospero negative cells were mainly positive for Stat-GFP expression. A small population of cells (white arrow) were small-sized and GFP positive but Delta-lacZ negative. Those could be undifferentiated progenitors like EBs. C- Arm\textsuperscript{+}/Pros\textsuperscript{-} small-sized stem and progenitor cells have Stat-GFP reporter activity. 5-day-old female flies were dissected and stained with α-Armadillo and α-Prospero antibodies. Scale bars: 10µm.
Figure 2.15
Figure 2.16 *Iduna* mutants have increased numbers of Stat-GFP positive cells. **A-** *Iduna* mutant clones had elevated Stat-GFP signaling in ISCs. 10xStat-GFP is a reporter for STAT signaling activity. *Iduna* mutant clones displayed strongly increased staining of this reporter. Posterior midguts of 7-day-old females were analyzed. **B-** The quantification of Stat-GFP reporter expression in *Iduna* mutant clones. GFP intensity was measured with Image J and normalized with control cells. *Iduna* mutant clones showed 4-5-fold higher reporter expression. **C-** *Iduna* mutants have more Stat-GFP positive ISCs and progenitors in midguts. Posterior midguts of 7-day-old females were analyzed. n>6 for each genotype. \( p<0.0001 \) is indicated as ****. **F-** Delta protein is elevated in the midguts of *Iduna* mutants. Posterior midguts of 7-day-old females were stained for Delta. Flies were collected and kept on regular diet at 24-25°C. Two-tailed Student’s *t*-test was used for statistical analyses. Error bars represented as means ± s.d. Scale bars: 10µm
Figure 2.17 Loss of *Iduna* activates the JAK-STAT pathway non-cell autonomously to promote ISC proliferation. **A**- *Iduna* mutants have elevated Stat-GFP signaling in ISCs. 10xStat-GFP is a reporter for STAT signaling activity. *Iduna* mutants displayed strongly increased GFP reporter. Posterior midguts of 7-day-old females were analyzed. **B**- The quantification of Stat-GFP expressing midgut cells. **C**- Knock-down of the *Stat92E* transcription factor in ISCs and EBs blocked ISC over-proliferation in *Iduna* mutants. In contrast, RNAi-mediated depletion of *Stat92E* in ECs did not affect proliferation of ISCs. 7-day-old female midguts were dissected and analyzed. **D**- *Iduna* depletion resulted in the upregulation of *upd3* mRNA expression in ECs. Myo1A driven *Iduna*-RNAi and *white*-RNAi expressing 7-day-old females were dissected for their midguts. Total RNA was isolated and cDNA libraries were prepared. *upd3* transcripts were amplified and analyzed by q-PCR. **E**- RNAi-mediated *Iduna* down-regulation induced *upd3>GFP* reporter activity. *Iduna* was knocked-down using RNAi driven by *upd3-Gal4*, and *GFP* was used as a reporter for *upd3* gene expression. ECs were stained with α-Armadillo antibody. EEs and ISCs were negative for *upd3>GFP* expression. *white*-RNAi served as a control. Posterior midguts of 3-day-old females were analyzed. **E**- Reduction of either *upd2* and *upd3* or their receptor *Domeless* suppressed over-proliferation of ISCs in *Iduna* mutants. Upon reduction of *upd2* and *upd3* gene dosage in *Iduna* mutants, I observed significantly less mitotic stem cells in *Iduna* mutants, comparable to wild type levels. Likewise, a 50% reduction of *Domeless* resulted in the suppression of ISC over-proliferation in *Iduna* mutants. On the other hand, these reductions in gene dosage of *upd2, upd3* and *Domeless* did not affect mitosis of ISCs in a wild type background. 7-day-old female midguts were quantified by pH3⁺ staining. Flies were fed with regular diet at 24-25°C. n>12 from each genotype. One-way ANOVA was used for statistical analyses. Error bars represented as means ±
s.d. Scale bars: 10\(\mu\)m. \(p < 0.0001\) was marked as ****. 

**G-** Model for the role of *Iduna* in the regulation of ISC proliferation. My model suggests that inactivation of *Iduna* causes Axin elevation which in turn decreases Wingless signaling activation in ECs and increases secretion of UPD cytokines from these cells. These cytokines activate JAK-STAT signaling through the Domeless receptor on neighboring ISCs and thereby induce ISC proliferation in the *Drosophila* midgut.
Figure 2.17

A. 

B. 

C. 

D. 

E. 

F. 

G.
Figure 2.18 RNAi-mediated *Iduna* depletion increases *upd3*->*GFP* reporter activity. A- Knock-down of *Iduna* results in upregulation of *upd3*. *Iduna* was down-regulated by two different UAS-RNAi lines (#36029 and #40882) under *upd3*-Gal4 driver. *upd3*->*GFP* was used as a reporter for *upd3* gene expression. *white*-RNAi served as control. B- C- *upd3* was upregulated in the ECs. Dissected midguts were stained with α-Armadillo and α-Prospero antibodies. ECs expressed *upd3*->*GFP* but EEs and ISCs did not. Posterior midguts of 3-day-old females were analyzed. Scale bars: 10µm.
Figure 2.18

A. upd3>GFP, White RNAi  upd3>GFP, Iduna RNAi  upd3>GFP, Iduna RNAi

B. upd3>GFP, Iduna RNAi  upd3>GFP, White RNAi

C. upd3>GFP  Armadillo/Prospero  Merged
2.4 Material and Methods

**Fly stocks:** Flies were kept at a 12-hour light/dark cycle. All crosses were performed at 22-25°C unless stated otherwise. The following fly stocks were used for this study (Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC) number (#) given in parentheses):

The stocks used in here: Df(3L)Exel6135 (BDSC# 7614), Df(3L)ED228 (BDSC# 8086), Df(3L)ED229 (BDSC# 8087), esg-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), esgK606 (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), 10xStat-GFP, Bach et al., 2007), UAS-GFP-Axin (BDSC# 7224), FRT82B, Axin044230 (a gift of Dr. Wei Du; Hamada et al., 1999), FRT82B, AxinE77 (a gift of Dr. Jessica Treisman; Collins and Treisman, 2000), Myo1A-Gal4, tub-Gal80ts, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006). The rest of Drosophila lines, which were studied here, were obtained from Steller Lab stocks. Oregon R flies were used as control and only adult female flies were analyzed in this study.

**Drosophila egg collection:** A 10cm² apple-agar plate was set up with embryo collection cage to provide a substrate for egg laying. Prior to adding the plate, a small quantity of yeast paste was smeared onto the center of the apple-agar. To provide moisture, water-soaked tissue paper was layered under embryo collection cages. 10-15-day-old adult flies were collected to cages, which
were then placed into a fly incubator at 24°C for 4 hours. Then, laden eggs were counted and 50 of them were plated into one corner of 10cm² apple-agar plates, in which had a straight yeast paste smear at the center. Agar plates finally were incubated in the incubator. After 24h, hatched eggs were counted.

To analyze larval development, hatched 1st instar larvae were counted and placed into a fresh agar plate with yeast paste until they were reached to the 3rd instar larvae. After counting, larvae were placed into vials containing regular food. They were counted both when they pupated and again when they eclosed.

5% sucrose diet: 5mm² Whatman filter papers were soaked with 1ml 5% sucrose solution and placed into the empty vials. The 5% sucrose solution was used as reduced nutrient diet. Eclosed adult females were collected at 24-25°C. When they were 2-day-old, their regular diet was replaced to 5% sucrose diet at 28°C. 20 of 2-day-old wild type or Iduna mutant female flies were grouped and kept on 5% sucrose solution-soaked filter paper containing vials at 28°C. Following the fly count, dead flies were removed and 1ml 5% sucrose-soaked filter papers were replaced daily.

Food intake measurement: Female flies of Iduna mutant and Oregon R were collected after they eclosed. Before measuring food intake, the flies were kept on the regular food for 6 days. The flies then transferred to the regular food supplemented with 0.5% (w/v) Acid Blue 9 (erioglaucine disodium salt, Sigma 861146) for 4 hours. Quadruplicates of 5 flies per sample were then homogenized in 250µl 1xPBS and cellular debris was removed by centrifugation. Food intake was quantified by measuring the absorbance of the supernatant at 630nm and normalized to the wet weight of the flies.
**CG8786/dRNFI46/dIduna CRISPR/Cas9 editing:** I used the CRISPR optimal target finder website (tools.flycrispr.molbio.wisc.edu/targetFinder) to identify an appropriate guide RNA (gRNA) target sequence within *dlduna* (Gratz et al., 2013, 2014). I purchased the forward 5'-GTCGCTAGCTGCAATCTGCTCTG-3’ and reverse 5’-AAACCAGAGCAGATTGCAGTAG-3’ oligos (IDT, Inc.) annealed, and followed the protocol; from Port et al., 2014 to clone the annealed oligos into pCFD3-dU6:3-gRNA plasmid (Addgene, plasmid# 49410, Port et al., 2014). Transformants were verified via Sanger sequencing (Genewiz, Inc.). The gRNA plasmid was injected into 300 embryos of custom *vasa-Cas9 Drosophila* (BestGene, Inc.). The injection was yielded 89 G₀ progeny, and I established 70 individual fly lines, a couple of which possible *Iduna* loss-of-function mutations.

**Isolation of the *Iduna* mutants and genetic mapping of *Iduna*–loss-of-function mutations:**
Total DNA was isolated from the 3ʳᵈ instar (L3) larvae or 5-day-old adults of *Iduna* homozygous mutants and controls by using the Roche genomic DNA extraction kit (Roche). To confirm the mutant line, PCR fragments were amplified with specific primers (forward primer 5’-CAGCCCGAGCTGGTCATACTCAG-3’, reverse primer 5’-CGGCTTTCTGGGCTACCTAC-3’) binding within 5’ UTR of *Iduna* and within the coding region of the gene. To identify the mutation site, the entire coding region was PCR amplified and PCR products were sent for DNA sequencing (Genewiz, Inc.).

**Cloning and generation of UAS-CG8786 transgenic *Drosophila***: Adult flies were directly homogenized in 1ml TRIzol (Life Technologies) and total RNA was isolated according to the manufacturer’s protocol. cDNA library was prepared from 5 μg total RNA, by using oligo(dT) amplification and the Superscript III First Strand synthesis kit (Invitrogen). cDNA library was used to amplify the *Iduna* transcripts with the primers (forward 5’-
ATGTCGCAACAGCGCTCCACAG-3’; *Iduna* B isoform reverse primer 5’-TCAGTAGAGCTTAGGTATACC-3’; *Iduna* C/G isoform reverse primer 5’-TCAGTAGAGCTTAGGTATACCG-3’). Amplified *dIduna* transcripts were cloned into pUAST (DGRC, Drosophila Genomic Resource Center) and pAc5.1 (Thermo Scientific) vectors by considering the appropriate restriction digestion sites. Following the bacterial transformation, all of the cloned genes were sequenced. To generate UAS-CG8786 transgenic *Drosophila*, Myc-tagged pUAST-CG8786/dIduna plasmid was injected into w1118 embryos (Best Gene, Inc.) We obtained successful transgenic lines.

**Total RNA isolation, cDNA synthesis and Q-PCR:** Posterior midguts of 7-day-old adult flies were directly homogenized in 1 ml TRIzol (Life Technologies) and total RNA was isolated according to the manufacturer’s protocol (miRNeasy mini kit, QIAGEN). cDNA library was prepared from 5 µg total RNA, by using oligo(dT) amplification and the Superscript III First Strand synthesis kit (Invitrogen). cDNA library was used to amplify *Upd3* and *Rp32l* transcripts with the forward 5’-AGGCCATCAACCTGACCAAC-3’, reverse 5’-ACGCTTCTCCATCAGCTTG-3’ and forward 5’-CCAAGGGTGATCGACAACAGA-3’, reverse 5’-CGATCTCGCCGCAGTAAAC-3’ primers, respectively. Those primers were designed via the online tool of DRSC/TRiP Functional Genomics Resources, Harvard Medical School and purchased from IDT, Inc.

**Cloning and generation of the wild type and mutants UAS-Flag-*Tnks* transgenic *Drosophila*:** I previously described *Drosophila* TNKS (Park and Steller, 2013) and its ORF was cloned into pUAST vector from pcDNA3.1-Flag-*Tnks*. To generate UAS-Flag-*Tnks* transgenic *Drosophila*, Flag-tagged pUAST-*Tnks* plasmid was injected into w1118 embryos (Best Gene,
Inc.) I obtained successful transgenic *Drosophila* lines and those were utilized in conjunction with tissue-specific *Gal4* drivers.

**Nile Red Staining**: 7-day-old female midguts and fat bodies were dissected in ice cold 1xPBS and kept on ice until required sample size obtained. Tissues were then fixed in ice cold 4% paraformaldehyde/PBS for 20 min at room temperature and washed with 1xPBS for 3x10 min. Tissues were stained with 0.25ug/ml Nile Red/PBS solution for 30 min at room temperature. Following the staining, samples were washed in 1xPBS for 3x10 min at room temperature. Specimens were finally mounted in Fluoromount-G (Southern Biotech) and analyzed with confocal microscope.

**Clone analyses and RNAi experiments**: Mutant clones were utilized to generate mitotic clones. 2nd instar larvae were subjected to an hour heat shock in a 37°C water bath per day until they reached the pupa stage and maintained at 24°C. 3-day-old adult females were analyzed.

For RNAi experiment, crosses were performed at 24°C and the progeny of the desired genotypes were collected on the day of eclosion and maintained at 24°C for 7 days before dissection. In the case of using temperature sensitive driver, eclosed virgin females were collected and kept at 29°C for 7 days before intestinal dissection.

**Cell culture**: S2R+ cells were maintained at 25°C in supplemented Grace’s Insect Medium (supplemented with 10% heat inactivated FBS, 100U/ml penicillin, 100µg/ml streptomycin) in spinner flasks.

**Development of polyclonal antibodies**: Full-length GST-tagged-Iduna C/G protein was expressed and purified from BL21 DE3 *E. coli* strain. Polyclonal antisera were generated in two guinea pigs (Cocalico, Inc.). For Western blot analyses, serum was used in 1:1000 dilutions.
**Western blot analyses**: 50-100µg dissected tissues or total larvae/flies were lysed in lysis buffer [50mM HEPES-KOH pH 7.4, 150mM NaCl, 0.05% Triton-X100, complete EDTA-free protease inhibitor cocktail (Roche)] using a 1 ml tissue grinder. Lysates were cleared by centrifugation at 13,000 g for 20 min at 4°C. Protein concentrations of supernatants were determined by BCA assay (Pierce). 1µg/µl lysate was prepared with 3X sample buffer in 100µl total volume (200mM Tris-HCl, pH 6.8, 200mM dithiothreitol (DTT), 8% SDS, 24% glycerol, 0.04% bromophenol blue), heated at 95°C for 10 min and samples were separated by SDS-PAGE for 1 h at 120V, by using standard 1X SDS Tris base-glycine running buffer. Proteins on the gels were blotted onto a PVDF membrane, in a 1X transfer buffer (25mM Tris base, 190mM Glycine, 20% MeOH, 0.05% SDS), and transferred (Bio-Rad) at 100V for 90 min. Membranes were taken through a standard immunoblot protocol followed by enhanced chemiluminescent detection (Crescendo ECL, Millipore) using a Lumimager (Fuji, LAS-3000). Primary antibodies: anti-Flag-HRP (1/1000, Sigma-Aldrich, A8592), anti-Flag (1/1000, CST, D6W5B 14793), anti-PAR (1/1000, Trevigen, 4335-MC-100), anti-Myc tag (1/1000, CST, 9B11, 2276), anti-Axin Drosophila (Santa Cruz, dT20, sc15685), anti-tubulin DM1A clone (1/1000, Sigma-Aldrich, T9026), mouse anti-GFP-HRP (1/2500, clone B2, Santa Cruz Biotechnology, sc-9996-HRP), rabbit anti-β-Actin-HRP (1/5000, clone 13E5, Cell Signaling Technology, 5125), donkey anti-rabbit-HRP (1/5000, Jackson ImmunoResearch, 711-035-152), donkey anti-mouse-HRP (1/5000, Jackson ImmunoResearch, 715-035-150), donkey anti-guinea pig-HRP (1:5000, Jackson ImmunoResearch, 706 006 148).

**Immunofluorescence**: Adult intestines were dissected in 1xPBS and fixed in 4% paraformaldehyde in PBS for 45 minutes at room temperature. Tissues then were first washed with 0.1% Tween 20-PBS, then washed with 0.1% TritonX-100-PBS and finally permeabilized
in 0.5% TritonX 100-PBS for 30 minutes. After blocking with 10% BSA in 0.1% Tween 20-PBS for 1 hour at room temperature, primary antibody incubation in 10% BSA in 0.1% Tween 20-PBS was performed for overnight at 4°C. 3X 5 min 0.1% Tween 20-PBS washed intestines then were then incubated in secondary antibodies for 1 hour at room temperature. Specimens were finally mounted in Fluoromount-G (Southern Biotech) and analyzed with confocal microscope (LSM780, Zeiss). Primary antibodies: mouse anti-Arm (Wang et al., 2016; N2 7A1, DSHB, 1:50), mouse anti-Prospero (Wang et al., 2016; MR1A, DSHB, 1/50), mouse anti-GFP (GFP-12A6, DSHB, 1/100), mouse anti-β-galactosidase (Tian et al., 2016); 401A, DSHB, 1/100), mouse anti-Delta (Wang et al., 2016; C594.9B, DSHB, 1/100), rabbit anti-phosho-S10-Histone3 (Wang et al., 2016; 06-570, Millipore, 1/1000). The secondary antibodies were goat anti-mouse-Alexa 488 plus (Thermo Fisher Scientific, A32723), goat anti-mouse-Alexa 568 (Thermo Fisher Scientific, A11031), goat rabbit-Alexa 546 (Thermo Fisher Scientific, A11035), goat rabbit-Alexa 488 (Thermo Fisher Scientific, A11034), goat anti-rabbit-Alexa 633 (Thermo Fisher Scientific, A21071) and used at 1/1000.

**Quantification of Stat-GFP immunostaining intensity:** Images from R5 region were taken with a 63x objective. Each Stat-GFP+ stem cell was identified using Imaris software (Bitplane). The main intensity in those cells within a field (40 μm x 40 μm) surrounding an Iduna mutant clone or an equal field at least 50 μm away from the mutant clone was measured. The relative intensity was calculated and shown in the figure (Wang et al., 2016). Statistical analyses were performed with Prism software (GraphPad).

**Immunoprecipitation:** S2R+ cells were seeded at 5x10^6 cells/10 cm^2 culture plates and incubated overnight at 25°C. Cells were then co-transfected with 5μg of each plasmid by using Mirus-insect transfection reagent. Negative controls were transfected with empty plasmids. 48
hours later, transfected cells were harvested. The cell pellets were washed within cold 1X PBS. This step was repeated 3 times. Pellets were re-suspended in 600µl 1% Triton X-100 lysing buffer. Re-suspended pellets were incubated on ice for 15 minutes and mixed gently and periodically. Total lysates were centrifuged at 13,000 rpm at 4°C for 30 minutes. The supernatant was removed and 100µl was stored as total lysate. 25µl Protein A/G (Thermo Scientific) beads were washed with lysing buffer for 3 times. 200µl of supernatant was incubated with the mixture of washed protein A-G beads on a rotator at 4°C for 30 minutes. In a parallel way, 25µl Protein A/G was washed with lysing buffer for 3 times. At the end of incubation period, the beads-supernatant mixture was centrifuged at 2,000 rpm at 4°C for 1 minute. Pre-cleaned supernatant was collected and added to beads. Antibody was added to supernatant-beads and incubated at 4°C on a rotator for 4 hours. The beads-supernatant-antibody mixture was centrifuged at 2,000 rpm at 4°C for 1 minute and beads were washed with lysing buffer for 3 times. In the final step, the beads were re-suspended in 50µl of 3X sample buffer to perform immunoblotting.

**Recombinant protein purification from S2R+ cells:** S2R+ cells were seeded at 5x10^6 cells/10 cm² culture plates and incubated overnight at 25°C. Then, Flag or Myc-tagged gene of interests were transfected and based on the small tag, a recombinant protein was immunoprecipitated with Flag or Myc agarose beads as described above. Finally, by using Flag or Myc peptides, tagged proteins were eluted and quantified by BCA (Pierce, ThermoFisher Scientific).

**Quantification and statistics:** ISC quantification, dissected midguts were stained with Armadillo and Prospero. Images of the R5 region (Buchon et al., 2013) were obtained with a 63X objective and total number of Arm⁺/Pros⁻ cells in a field were counted. Quantifications of immunoblot were done with Image J. Two-tailed Student t-test and One-way ANOVA were used as statistical analyses and those were done with Prism (GraphPad) software.
3  *In vivo* functional analyses of lysine 598 in *Drosophila* TNKS

3.1 Summary

ADP-ribosylation is a strikingly dynamic and reversible post-translational modification. TNKS is conserved from flies to human. Although auto-poly-ADP-ribosylation is a degradation signal for TNKS, regulation of its poly-ADP-ribose activity is still a mystery. The ADP-ribosylation sites in TNKS have not been mapped to the specific residue(s) yet. Iduna is a binding partner of TNKS. Iduna-dependent TNKS degradation requires its PARsylation. My work revealed that lysine 598 is an ADP-ribosylation site in *Drosophila* TNKS. *Tnks<sup>K598A</sup>* knock-in flies live significantly shorter compared to control flies. Strikingly, *Tnks<sup>K598A</sup>* adult flies exhibit climbing deficits with age. *Tnks<sup>K598A</sup>* flies reduced the levels of phosphorylated JNK and poly-ADP-ribosylated proteins. Unlike *Tnks*-null mutants, *Tnks<sup>K598A</sup>* does not cause hyper-proliferation of stem cells in the adult midgut. I finally identified that K598A mutation prominently reduces the binding of certain proteins. As a result, I suggest that K598 residue is essential for building up the interaction between TNKS and certain partners.
3.2 Introduction

ADP-ribosylation is a post-translational modification (PTM) that regulates many essential biological functions including DNA repair, chromatin structure, unfolded protein response, and apoptosis. ADP-ribose transferases (ARTs) attach ADP ribose (ADP-r) units to acceptor proteins by using NAD+. Some ARTs can subsequently add more ADP-r units to target proteins to generate poly-ADP-ribose (PAR) chains. Glutamic acid, aspartic acid, lysine, arginine, cysteine, or phosphorylated serine amino acid residues can be modified by ARTs (DaRosa et al., 2018; Martello et al., 2016; Zhang et al., 2013).

PAR moieties are negatively charged polymers. Although poly-ADP-ribosylation (PARsylation) has an estimated half-life of only 1-6 min, it may influence the fate of proteins through several mechanisms, including a direct effect on protein activity, stability, or recruitment of binding partners (Guettler et al., 2011). Several enzymes can reverse protein ADP-ribosylation. NUDIX9 and NUDT16 process both mono-ADP-ribose (MAR) and PAR units. Impairments in the hydrolysis of PAR chains involve in various disease conditions such as cancer, neurodegeneration, oxidative stress, neural injury, and regeneration (Deng, 2009; Hanai et al., 2004; Martire et al., 2015; Brochier et al., 2015). Therefore, protein PARsylation and its regulation are essential.

Tankyrases (TNKS/PARP5) have essential roles in cellular pathways including telomere length maintenance, Notch signaling, and Wnt signaling (Bhardwaj et al., 2014). TNKS contains catalytic PARP domain, α-steril oligomerization motif (SAM) and large ankyrin repeat clusters (ARCs), known for substrate recognition and binding (Smith et al., 1998; De Rycker and Price, 2004). Although SAM-dependent polymerization strikingly enhances its PARP activity, auto-
PARsylation inhibits TNKS polymerization (De Rycker and Price, 2004; Nottbohm et al., 2007; Fan et al., 2018).

The fly genome has a highly conserved Tnks gene. Loss-of Tnks is not required for viability in Drosophila. TNKS-mediated ADP-ribosylation activates Wingless signaling (Croy et al., 2016; Feng et al., 2014; Yang et al., 2016; Wang et al., 2016). Loss-of Tnks, therefore, causes Axin elevation that blocks Wingless signaling in enterocytes and activates JAK-STAT signaling to promote stem cell division in the fly midgut (Wang et al., 2016). On the other hand, the conditional depletion of Tnks1/2 in mice results in a rapid decrease of Lgr5+ stem cells and promotes stem cell death in the small intestine. As a result, mortality of double knockout mice increases (Ye et al., 2018).

Here, I showed that Iduna is a TNKS-binding protein that plays an important role in TNKS degradation after its auto-PARsylation. Using a mass spectrophotometry-based approach, I identified lysine 598 as an ADP-ribosylation site in Drosophila TNKS. I then generated TnksK598A knock-in flies by using CRISPR-Cas9 genome engineering to investigate in vivo function(s) of K598 residue in Drosophila. Finally, I found that 11 interactors of TNKS have considerably reduced binding to the K598A mutant. This points to a critical function of K598 in mediating interactions between TNKS and various binding partners. In the future, it will be interesting to determine the functional consequences of these interactions.
3.3 Result

3.3.1 Iduna interacts with TNKS and plays a role in TNKS degradation in *Drosophila*

TNKS is a well-defined substrate of Iduna both in both vertebrates and *Drosophila*. Auto-PARsylation of TNKS is a degradation signal (Zhang et al., 2011). Loss-of Iduna leads to elevation of mis-expressed TNKS in *Drosophila* eye (Gultekin and Steller, 2019). However, it is still a mystery of how TNKS is rapidly destructed. To understand TNKS degradation, I focused on PARsylation and Iduna-mediated ubiquitylation of TNKS.

Iduna recognizes and ubiquitylates the PARsylated proteins through its WWE and RING domains, respectively (Fig 3.1A). I deleted the functional RING, WWE, and PAR domains to determine the structure and functions of Iduna. These mutants were then co-expressed with TNKS in *Drosophila* S2R+ cells. Wild type Iduna led to degradation of TNKS while the mutants did not led destruction of TNKS, resulting in elevated protein levels of TNKS in cells (Fig 3.1B). This confirmed that Iduna plays a role in TNKS degradation.

TNKS recognizes peptide motifs, which consist of six minimal consecutive amino acids. The canonical TNKS-binding motif (TBM) is Rxx#DG that starts with arginine in the 1\textsuperscript{st} position and ends up glycine as the 6\textsuperscript{th} amino acid residue whereas x can be any amino acid, and # is a small hydrophobic amino acid (DaRosa et al., 2018). Iduna has a putative TNKS binding motif, RATEDG, in the WWE domain (Fig 3.1C). To test if this motif was necessary for TNKS binding, Flag-TNKS was immunoprecipitated with wild type or a motif deficient mutant of Iduna in S2R+ cells. The deletion mutants were also co-immunoprecipitated with TNKS (Fig 3.1D).
Figure 3.1 *Drosophila* Iduna interacts with TNKS and ubiquitinylates *Drosophila* TNKS. A- Iduna has evolutionarily conserved WWE, PAR, and RING functional domains, responsible for the degradation of its ADP-ribosylated target proteins. Truncation mutants of Iduna were generated to conduct structure-function analyses in S2R+ cells. B- Truncation mutants of Iduna prevented *Drosophila* TNKS degradation. TNKS was co-expressed with wild type or the truncation mutants of Iduna in S2R+ cells. Labeled proteins were detected by immunoblotting. C- Iduna has several putative TNKS-binding motifs (TBMs): On the RING domain RLPCG, and RATEDG on the WWE configuration. D- Iduna interacts with TNKS. Flag-TNKS was co-expressed with wild type or the truncation mutants of Iduna in S2R+ cells. Co-IP was done with anti-Flag, and WB was conducted with anti-GFP and anti-Flag. E- Iduna was co-localized with TNKS in S2R+ cells. *UAS-RFP-Tnks* was co-expressed with *UAS-AcGFP-Iduna* under Act5C driver in S2R+ cells. F- Iduna ubiquitylated TNKS in S2R+ cells. Flag-TNKS was co-expressed and AcGFP- wild type or Iduna mutants in S2R+ cells. After 24h, S2R+ cells were treated with 5µM Bortezomib. 10h later, cells were collected and lysed. HA-conjugated A/G beads were used to pull down Flag-TNKS. Western blotting was performed with an anti-HA antibody. G- Ubiquitin ligase activity of Iduna depended on 141-Histidine and 147-Cysteine residues in the RING domain. H- Ubiquitin ligase deficient Iduna did not degrade TNKS. Flag-TNKS was co-expressed with AcGFP- mutant or wild type Iduna in S2R+ cells. Labeled proteins were analyzed by immunoblotting. β-actin was a loading control.
Figure 3.1

A

WT_Iduna

Δ-RING_Iduna

Δ-WWE_Iduna

Δ-PAR_Iduna

B

GFP-Iduna:
Flag-TNKS: + + + + + + - - - - - -

Flag-TNKS:
WB α-flag

AcGFP-Iduna
WB α-GFP

β-Actin

D

AcGFP-Iduna:
Flag-TNKS: + + + + + + - - - - - -

IP: α-flag
WB: α-GFP

IP: α-flag
WB: α-GFP

WB: α-GFP

IP: whole
cell
lysate

E

Drosophila S2R+ cells

Act5C Gal4>
UAS-RFP-TNKS

Act5C Gal4>
UAS-GFP-Iduna

dlduna Putative TNKS binding motifs

F

AcGFP-Iduna
Flag-TNKS + + + +
HA-Ubiquitin - - - -
Bortezomib + + + +

IP: α-Flag
WB: α-HA

H

AcGFP-Iduna:
Flag-TNKS:
WB α-flag

WB: α-HA

β-actin

Table: dlduna RING domain

hIDUNA
mIDUNA
dmIDUNA-PB
dmIDUNA-PC
dmIDUNA-PF
daeIDUNA-PF

VPECAI CLQCVHVP SLFPCMVPC TC CVXGASH
VPECAI CLQCVHVP SLFPCMVPC TC CVXGASH
ALECPI CLQCHPRLPCM PCPFC CVXGAY
ALECPI CLQCHPRLPCM PCPFC CVXGAY
ALECPI CLQCHPRLPCM PCPFC CVXGAY
ALECPI CLQCHPRLPCM PCPFC CVXGAY

141 147

96
Figure 3.2 TNKS degradation requires its poly-ADP-ribosylation and Iduna plays a role in TNKS degradation in vivo. A- TNKS ADP-ribosylates its target proteins through its PARP domain. B- M1064, H1041, and E1148 are the catalytic PARP residues, conserved from flies to human. C- Alanine mutations on these residues led to a deficiency in auto-poly-ADP-ribosylation (PARsylation). Mutant proteins were purified from Drosophila S2R+ cells by using Flag-beads. D- TNKS auto-ADP-ribosylation is necessary for its Iduna-dependent degradation. Wild type Drosophila TNKS or the PARP deficient M1064V, H1041A, E1148A mutants of TNKS were co-expressed with and without Iduna in S2R+ cells. E- The small molecule inhibitor of TNKS, XAV939 blocks the auto-ADP-ribosylation of TNKS that leads to TNKS elevation in S2R+ cells. Transfected S2R+ cells were treated with 20µM XAV939. Ribosylated TNKS proteins were detected by anti-PAR immunoblotting after TNKS-immunoprecipitation. β-actin was a loading control. F- RNAi-mediated Iduna-depletion causes an elevation of miss-expressed TNKS in Drosophila. UAS-HA-Tnks transgene was expressed under the temperature sensitive Tub>Gal4 driver. Iduna inactivation by RNAi led to TNKS accumulation. 7-day-old female flies were lysed. Total proteins were analyzed by Western blotting. white-RNAi was a negative control. F- Quantification of Western blotting. The experiment was repeated two times with two independent replicates. Lines indicated means ± s.d. (standard deviation) ***p<0.001 Statistical analyses were done by two-tailed Student’s t-test. H- Iduna-RNAi caused an accumulation of PARsylated TNKS in Drosophila midguts. UAS-Iduna RNAi or white-RNAi and UAS-HA-Tnks transgenes were expressed under the temperature sensitive Tub>Gal4 driver. 7-day-old female flies were dissected and stained with α-PAR, α-HA and DAPI.
Figure 3.2

A

Flag-TNKS: WT, H104A, H104A, E116A

pADPR-TNKS
WB: α-PAR

WB: α-FLAG

@ 37°C

B

PARP domain

<table>
<thead>
<tr>
<th>dTNKS</th>
<th>hTNKS</th>
<th>mTNKS</th>
<th>mTNKS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERLFGG</td>
<td>ERLFGG</td>
<td>ERLFGG</td>
<td>ERLFGG</td>
</tr>
<tr>
<td>MFMAHA</td>
<td>MFMAHA</td>
<td>MFMAHA</td>
<td>MFMAHA</td>
</tr>
<tr>
<td>GGLHFAE</td>
<td>GGLHFAE</td>
<td>GGLHFAE</td>
<td>GGLHFAE</td>
</tr>
</tbody>
</table>

D


Flag-TNKS: WT, H104A, E116A

HA-Iduna: +, +, +, +, +, +

kDA

170

130

100

70

55

35

F

Tub Gal4>>

RNAi: wild type, Iduna RNAi, Iduna RNAi

HA-Tankrase

β-Actin

G

Relative HA-TANK protein level

Drosophila adult midgut
That suggests the interaction between Iduna and TNKS is independent of ADP-ribosylation and ubiquitylation. Therefore, Iduna has more than one TBMs, one of which is sufficient to bind TNKS. In addition to immunoprecipitation, Iduna was co-localized with TNKS in S2R+ cells (Fig 3.1E). Taken together, TNKS can work together with Iduna to control the degradation of its substrates.

I next focused on ubiquitin ligase activity of Iduna. I first showed that Iduna ubiquitylated TNKS by conducting in vivo ubiquitylation in S2R+ cells (Fig 3.1E). I then characterized the RING domain of Iduna. Histidine and cysteine residues in the RING domain are responsible for ubiquitin ligase activity in both mouse and human Iduna (Kang et al., 2011). Both residues are conserved in Drosophila (Fig 3.1F). I mutated both residues into alanine to test their ubiquitin ligase functions. Each mutant (H141A or C147A) partially led to TNKS degradation compared to the wild type Iduna (Fig 3.1G). However, the H141/C147-AA mutant was completely unable to degrade TNKS (Fig 3.1H). As a result, TNKS degradation requires Iduna-dependent ubiquitylation.

3.3.2 Poly-ADP-ribosylation is essential for TNKS degradation

Auto-ADP-ribosylation is crucial to control TNKS stability. Auto-PARsylation is catalyzed by PARP domain (Fig 3.2A), which is 86% similar to its corresponding amino acid sequences in mouse and human TNKS. Mammalian PARP of TNKS has several catalytic amino acid residues including M1064, H1041, and E1148 (Callow et al., 2011). These residues are conserved in Drosophila (Fig 3.2B). Recombinant Flag-M1064A, H1041A, and E1148A TNKS were deficient for auto-PARsylation (Fig 3.2C).

Unlike wild type TNKS, these TNKS mutants were also resistant to degradation in the presence of wild type Iduna in S2R+ cells (Fig 3.2D-E). Auto-PARsylation of TNKS is
necessary for its stability. As a proof of concept, a small molecule inhibitor of TNKS, XAV939, blocked its auto-PARsylation as well as Iduna-dependent-degradation. As a control, the PARP deficient H1041A mutant had no auto-PARsylation. Therefore, it was stable to Iduna-dependent degradation (Fig 3.2E). RNAi-mediated *Iduna* inactivation also led to an elevation of miss-expressed TNKS *in vivo* (Fig 3.2F-G). *Iduna* knock-down resulted in an accumulation of PARsylated TNKS in the *Drosophila* midgut (Fig 3.2H). These results together demonstrate that ADP-ribosylation is necessary for degradation of TNKS in *Drosophila*.

### 3.3.3 Lysine 598 (K598) is an ADP-ribosylation residue in *Drosophila* TNKS

ADP-ribosylation is a highly dynamic PTM with an estimated half-life of only 1-6 min (Palazzo et al., 2015). Although auto-PARsylation is essential for TNKS degradation, the ADP-ribosylation site(s) in TNKS have not been mapped to the specific residue(s) yet. To this end, I developed a mass spectrophotometry (MS) approach to identify the amino acid residue(s) in *Drosophila* TNKS (Fig 3.4A).

Since human NUDT16 (hNUDT16) removes both poly- and mono-ADP-ribosylation, hNUDT16 cleavage can be a tool to trim the complex protein ADP-ribosylation into an easily detectable mark by mass spectrometry (Palazzo et al., 2015). To test if hNUDT16 could exhibit phosphodiesterase activity against PARsylated TNKS, I first expressed *Drosophila* TNKS in *Drosophila* S2R+ cells and hNudt16 in *E. coli*. Subsequently, I purified both Flag-tagged dTNKS and His-tagged hNUDT16 proteins by using cobalt and Flag conjugated beads, respectively (Fig 3.3A-B). I first PARsylated TNKS *in vitro* for 1 hour (Fig 3.4B, lane 1) and then incubated the purified hNUDT16 with PARsylated TNKS for 3 hours. Strikingly, the recombinant hNUDT16 showed significant ability to remove the PAR signal from TNKS (Fig
4.3B). I further tested the range of hNUDT16 concentrations by PAR hydrolysis assay (Fig 3.4B, lanes 2-6). PAR modification was visualized by Western blot, using an antibody specifically recognizing poly- and oligo chains of ADP-r but not MAR. I detected molecular shifts of TNKS with and without modification in an hNUDT16-concentration-dependent manner in vitro. hNUDT16, therefore, efficiently hydrolyzes PAR modification in TNKS.

In my MS approach, following in vitro ADP-ribosylation, PARsylated Drosophila TNKS was cleaved by hNUDT16, leaving a ribose-5’-phosphate (R5P) footprint of ADP-ribosylation in target amino acids. After trypsin digestion, R5P-containing peptides were enriched via TiO₂ pull down and analyzed by MS (Fig 3.4A). With MS, R5P can be detectable in the peptides of interest (Palazzo et al., 2015). I identified several peptides, having R5P (Fig 3.4C). However, only one peptide, GKYDICK, had an R5P molecular marker with an expected molecular size (Fig 3.5A). I identified K598 residue as one of the ADP-r acceptor sides in TNKS (Fig 3.5B). K598 is the first investigated PARsylation site in TNKS.

K598 localizes in the ARC4 repeat of Drosophila TNKS (Fig 3.4D and Fig 3.6A-B). I first aligned the amino acid sequences of TNKS from different species and found that K598 is conserved in evolution. Both human and mouse orthologs of Drosophila TNKS shares high identity with ARC4 (Fig 3.4D) suggesting that K598 may have a conserved function. I then mutated lysine 598 to alanine (K598A) to examine its PARP activity. I purified Flag-K598A TNKS from S2R+ cells and carried out ADP-ribosylation assays. Strikingly, the K598A mutation completely blocked auto-PARsylation of TNKS in vitro just like PARP-deficient TNKS M1064V, H1041A and E11418A mutants (Fig 3.4E).
Figure 3.3 Expression and purification of recombinant *Drosophila* TNKS and human NUDT16. **A**- *UAS-Flag-TNKS* was expressed under *Actin5C-Gal4* driver in S2R+ cells. After 48h, transfected S2R+ cells were lysed to purify Flag-tagged TNKS by Flag-antibody conjugated beads. Purified TNKS was stained with colloidal blue on the SDS-poly-acrylamide gel. **B**- Expression and purification of His-tagged *hNudt16* from *E. coli* after IPTG induction. Cobalt beads were used to purify the His-tagged protein. Colloidal blue staining was used to visualize the purified proteins.
Figure 3.4 Mass spectrophotometric approach identifies ADP-ribose acceptor residues of TNKS. A- I had developed an MS-based approach to identify ADP-ribose acceptor residues in TNKS. After in vitro ADP-ribosylation of TNKS, human NUDT16 removed PARsylation in TNKS. B- Human NUDT16 cleaves the poly-ADP-ribose modification in TNKS in vitro. By using NAD+, purified TNKS auto-poly-ADP-ribosylated in vitro (Lane 1). Recombinant hNUDT16 removed the PAR polymers from TNKS. Flag-TNKS was purified from S2R+ cells. For 3h, hNUDT16 digested PAR units in TNKS in a concentration-dependent manner (Lanes 2-6). C- hNUDT16 left a small molecular footprint, ribose-5′-phosphate (R5P) in TNKS. TiO2 pull down enriched R5P-containing peptides, which were finally detected by mass spectrophotometry (MS). GKYDICK peptide in Drosophila TNKS has a ribose-5′-phosphate molecular marker. D- K598 residue is conserved from flies to human. K598 residue in Drosophila TNKS corresponds to K604 in human TNKS2. E- K598A mutant TNKS did not PARsylated by itself in the presence of NAD+ in vitro. TNKS proteins were expressed and purified from S2R+ cells. PARP deficient TNKS mutants (M1064V, H1041A, and E1148A) were also tested in the ribosylation assay. The modification was detected with an anti-PAR antibody. Unmodified TNKS was recognized by a Flag-immunoblotting. F- Drosophila TNKS PARsylated V5-Axin through TNKS-binding motif (TBM) in vitro. On the other hand, TNKS did not modify TBM deleted V5-Axin. Flag-TNKS and V5- wild type Axin or V5-D-TBM-Axin were expressed and purified from S2R+ cells. ADP-ribosylation was conducted in vitro with the purified proteins from S2R+ cells. G- K598A mutant was unable to modify GFP-Axin in vitro. GFP-Axin was expressed under the temperature sensitive Tub-Gal4 driver and purified from Drosophila. H- K598 in Drosophila TNKS corresponds to K604 in human TNKS2. TBM of Axin is conserved from flies to human. Glutamic acid is a negative and lysine is a positive charged amino acid. Hence, the 7th
and 8\textsuperscript{th} glutamic acid residues in the TBM of human AXIN can form a strong stable salt bridge interaction with K604 in human TNKS2.
Figure 3.5 The GKYDICK peptide in *Drosophila* TNKS has R5P, detected by MS. A- The peptide has an expected molecular mass with R5P modification. The K598 in the GKYDICK peptide was identified as one of the ADP-ribose acceptor residues. B- Lysine 598 is an ADP-ribose acceptor residue in *Drosophila* TNKS.
Figure 3.5

A

GKYDICK

B

GKYDICK, K2-Ribose-5-phosphate_neutral (212.00859 Da), C6-Carbamidomethyl (57.02146 Da) Charge: +2,
Monoisotopic m/z: 548.22559 Da (+0.53 mnu/+0.97 ppm),
MH+: 1095.44390 Da, RT: 27.27 min, IonScore:17
Figure 3.6 Lysine 598 is one of the ADP-acceptor residues in *Drosophila* TNKS. A-Lysine 598 localizes in the ARC4 repeat of *Drosophila* TNKS, that is conserved during evolution. B-K598 in Drosophila TNKS is corresponding to K604 residue in human TNKS. C- *Drosophila* Axin has an N-terminus TBM. Axin is a scaffold protein that recruits and interacts with different proteins through its various domains.
Axin, the rate-limiting factor in the Wingless pathway, is a target protein of TNKS in *Drosophila* as well. Axin also functions as a scaffold as it recruits different proteins through its various domains. It interacts with Adenomatous Polyposis Coli (APC) protein via its RGS domain and also binds to Shaggy (GSK3 in mammals) and Armadillo (β-catenin in mammals) through its BCD domain. TNKS recognizes and binds to Axin through its TNKS-binding motif (TBM) (Fig 3.6C).

I demonstrated that TNKS was unable to PARsylate Axin when its TBM was mutated. Hence, TBM is necessary for TNKS-dependent PARsylation of Axin (Fig 3.4F). I next tested PARP activity of K598A on its substrate Axin. I purified GFP-tagged Axin from flies and conducted *in vitro* ADP-ribosylation assay with wild type, K598A, and M1064V mutants of TNKS. Wild type TNKS PARsylated GFP-Axin as well as itself (Fig 3.4G). However, both K598A and M1064V TNKS mutants were unable to modify GFP-tagged Axin in this *in vitro* assay (Fig 3.4G). These results suggest that K598A may be a loss-of-function mutation of TNKS.

K598 residue in *Drosophila* TNKS corresponds to K604 in human TNKS2 (Fig 3.4H). Independent of my investigation, K604 residue in human TNKS2 was shown to be essential for AXIN1 interaction (Guettler et al., 2011). Based on fluorescence polarization peptide binding assays, the binding of TBM in AXIN1 was indeed highly sensitive to the K604A mutant peptide of TNKS2 (Guettler et al., 2011). It suggests that the position 7 and 8-glutamic acid residues in the TBM of AXIN can form a robust stabilizing salt bridge interaction with K604 of TNKS2 (Guettler et al., 2011). Taken together, I suggest that K598 residue may be necessary for ADP-ribosylation of Axin in *Drosophila*.
Figure 3.7 Tnks\textsuperscript{K598A} knock-in mutation is introduced into Drosophila Tnks gene by CRISPR-Cas9 genome engineering. A- A specific sgRNA was designed to target K598 residue. To mutate lysine residue to alanine, single-stranded DNA was designed and injected together with the sgRNA into Cas9-expressing embryos. B- I first screened the established fly lines with PCR and Styl-RFLP since K598A mutation changed one of the Styl restriction enzyme recognition sites. Several Drosophila lines were identified as K598A mutant, whose PCRs failed to give 2 DNA fragments (328 and 308 nucleotides). C- Identified Tnks\textsuperscript{K598A} knock-in mutants by Sanger sequencing. Corresponding Tnks sequences from wild type, heterozygous and homozygous flies were compared for K598 codon. D- Tnks\textsuperscript{K598A} mutants are viable, fertile and do not have obvious morphological defects. Tnks\textsuperscript{K598A} knock-in mutants did not have defects in hatching their embryos. n>200 for each genotype. E- Tnks\textsuperscript{K598A} knock-in mutants did not have defects in eclosing their pupae. n>100 for each genotype. Each line indicated means ± s.d. Statistical analyses were done with two-tailed Student’s \textit{t}-test.
Figure 3.7

A

*genome cleavage site*  
PAM sequence  
GCAATATCATACTTTTCCCT TGG (+strand)  
sgRNA  

tnks genomic locus  

WT ...GAA GCT GCC GCC AAG GGA AAG TAT ...  
K598A ...GAA GCT GCA GCA AAG GGA GCG TAT ...  
single strand donor DNA

B

- Styl  
+ Styl  

PCR  
Styl RFLP  

Styl DNA fragments (bp)  

C

GAAGCTGCGGCCAAGGAAATAT  

D

Hatched embryo %  

E

Eclosed pupae %  

ns  

Wild type  
Tnks K598A/K598A
To investigate the physiological function(s) of K598 residue in *Drosophila*, I generated *Tnks*<sup>K598A</sup> knock-in mutant, in which the lysine 598 residue in *Tnks* gene was replaced with an alanine residue (Fig 3.7A). Both sgRNA and donor DNA injected into Cas9-expressing embryos. I finally established possible *Tnks*<sup>K598A</sup> knock-in fly lines. After Styl restriction fragment length polymorphism (RFLP) analyses and complementary Sanger sequencing (Fig 3.7B), I identified *Tnks*<sup>K598A</sup> mutant flies (Fig 3.7C).

3.3.4 *Tnks*<sup>K598A</sup> knock-in mutation reduces lifespan and JNK signaling in *Drosophila*

I first examined the larval development of *Tnks*<sup>K598A</sup> and Oregon R flies but did not observe any differences in the numbers of hatched embryos (Fig 3.7D), pupated larvae, and eclosed adult *Drosophila* (Fig 3.7E), between *Tnks*<sup>K598A</sup> mutants and wild type flies. Similar to *Tnks*<sup>L19</sup> and *Tnks*<sup>S03</sup>-null mutants, *Tnks*<sup>K598A</sup> adult flies are viable, fertile, and have no apparent morphological defects when compared with wild type flies (Wang et al., 2016). Strikingly, both K598A females and males had significantly shorter lifespans compared to control flies (Fig 3.8A-B). Surprisingly, *Tnks*<sup>K598A</sup> adult flies reduce their climbing behavior with age (Fig 3.8C, Fig 3.9A-C). *Tnks* knock-out flies also have prominently shorter lifespan and impaired climbing ability due to the JNK pathway (Li et al., 2019). To address if K598 residue was important for JNK signaling in *Drosophila*, I analyzed the protein levels of phosphorylated JNK (p-JNK) and indicated that *Tnks*<sup>K598A</sup> flies have less p-JNK proteins when they were 4-week old compared to their controls (Fig 3.8D-E). Only two PARP family members, TNKS and PARP-1, exist in *Drosophila* and modify their substrate proteins via PARsylation (Krishnakumar and Kraus, 2010). Consistently, the levels of PARsylated proteins were reduced in the *Tnks*<sup>K598A</sup> mutants compared to their controls (Fig 3.8F-G).
Figure 3.8 *Tnks*<sup>K598A</sup> mutation significantly reduces lifespan in *Drosophila*. A- K598A females and B- males had significantly shorter lifespan than control flies. Both females and males *Tnks*<sup>K598A</sup> mutants were independently analyzed on a regular diet at 24-25°C. For statistical analyses, I used the Mantel-Cox and Gehan-Breslow-Wilcoxon tests to compare survival curves between *Tnks*<sup>K598A</sup> mutants and control flies. n>100 for each genotype. C- *Tnks*<sup>K598A</sup> adult flies reduced their climbing abilities with age. *Tnks*<sup>K598A</sup> mutation did not affect climbing behavior in flies when they were 3-day-old (Week 0). n>100 for each genotype. Female flies were tested for their climbing for 4 weeks. n>100 for each genotype. D- *Tnks*<sup>K598A</sup> flies have less phosphorylated-JNK proteins when they were 4-week old compared to their controls. Whole fly protein lysates were used in Western blot analysis. p-JNK levels were detected by antibody. E- Quantification of p-JNK levels. Normalization was done with α-Tubulin. F- Protein PARsylation was decreased in the *Tnks*<sup>K598A</sup> mutants compared to their control. PARsylation was detected by α-PAR antibody. G- Decline in the global PARsylation was quantified and normalized with α-Tubulin. Each line indicated means ± s.d. Statistical analyses were done with two-tailed Student’s *t*-test or 2-way ANOVA. ****p< 0.0001 for C.
Figure 3.9 Tnks<sup>K598A</sup> mutation significantly reduces their climbing behavior in Drosophila.

A- Tnks<sup>K598A</sup> mutation did not affect climbing behavior in flies when they were 3-day-old. n>100 for each genotype. B- Tnks<sup>K598A</sup> adult flies reduced their climbing abilities with age. 45-day-old females were tested for their climbing. n>100 for each genotype. C- Image from 45-day-old female flies during their climbing assay. n=21 for each genotype. D- TAG levels did not change in Tnks<sup>K598A</sup> mutants during aging. Quantification of total triglycerides (TGA). Each line indicated means ± s.d. Statistical analyses were done with two-tailed Student’s t-test.

****p< 0.0001 for B.
Figure 3.10 *Apc1, pygo, and Arm* are indispensable for Wingless-dependent regulation of stem cell proliferation in the adult midgut. **A**- FRT-RFP was a control mutant clones, which did not promote the proliferation of *esg-LacZ*+ stem and progenitor cells. **B**- *Apc1* is required for ISC hyper-proliferation in adult fly midguts. FRT82B, *Apc1* is an *Apc1*-null mutant. **C**- Wingless co-transcription factor *pygo*-null mutants had an increased number of *esg-LacZ*+ cells. **D**- Armadillo is essential to prevent over-proliferation of stem cells. FRT19A, *arm* is *arm*-null mutant. Posterior midguts from 7-day-old *FRT-RFP* animals were dissected and stained with α-β-gal. *FRT-RFP* mutant clones did not have RFP signals. These flies carried *esg-lacZ* reporter in their backgrounds.
Figure 3.11 **Tnks prevents the over-proliferation of ISCs.**

A- *Tnks*\(^{503}\) mutant clones had increased number of Arm\(^{+}/\)Pros\(^{-}\) stem cells compared to FRT82B control clones in the *Drosophila* midgut. Posterior midguts from 7-day-old *FRT*-RFP animals were dissected and stained with a-Armadillo and a-Prospero antibodies. Armadillo stained cell membranes.

B- *Tnks* deletion in mutant clones resulted in promotion of *esg*-GFP\(^{+}\) stem and progenitor cell numbers in adult midguts compared to FRT82B control clones. Posterior midguts from 7-day-old *FRT*-RFP flies were analyzed. Flies had *esg*-GFP in their backgrounds. Mutant clones were defined by the absence of an RFP signal. Scale bar: 10µm.
Figure 3.11

A

<table>
<thead>
<tr>
<th>Armadillo/Prospero</th>
<th>RFP NLS</th>
<th>DAPI</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT82B, control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRT82B, Trks^{03}</td>
<td></td>
<td></td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>esg&gt; GFP</th>
<th>RFP NLS</th>
<th>DAPI</th>
<th>Merged</th>
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<tr>
<td>FRT82B, control</td>
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<td>FRT82B, Trks^{03}</td>
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Figure 3.12 *Tnks* is essential in ECs to prevent hyper-proliferation of midgut stem cells. A- TNKS is crucial to control Axin levels in the ECs. Inactivation of *Tnks* elevated mis-expressed Axin levels in adult midguts. GFP-Axin transgene was mis-expressed in ECs under the *Myo1A* driver. 5-day-old flies were dissected. Midguts were lysed to conduct Western blotting. Tubulin was used as a loading control. B- *Tnks* mutants had more *esg-LacZ* stained midgut stem cells and progenitors. C- *Tnks* deletion promotes the number of *esg>GFP* cells. D- Ectopic expression of *UAS-Flag-Tnks* transgene in ECs suppressed the increased number of ISCs in *Tnks* mutants. *Myo1A-Gal4* was used to express *Tnks* in ECs. Arm+ /Pros− stem cells were quantified from posterior midguts. E- Quantification of pH3− cells in whole midguts. n>5. Both *Tnks*19/19 and *Tnks*503/503 are *Tnks*-null flies. Heterozygous flies (*Tnks*503/+ or *Tnks*19+/+) were used as control flies. Each dot represented an animal and lines indicated means ± s.d. ****p<0.0001. Statistical analyses were done with One-way ANOVA.
Decrease in the global PARsylation suggested that \( Tnks^{K598A} \) mutants might be a defect in energy storage. To test that, I examined levels of total triglycerides (TGA). However, I found that TAG levels were not altered in these mutants during aging (Fig 3.9D). I, therefore, reasoned that \( Tnks^{K598A} \) might be a hypomorph of \textit{Drosophila} TNKS.

My previous \textit{in vitro} results suggested that \( Tnks^{K598A} \) might be a loss-of-function mutant that might lead to Axin elevation and the promotion of stem cell proliferation in \textit{Drosophila}. To test this, I analyzed midguts of both \( Tnks \)-null and \( Tnks^{K598A} \) mutants. As indicated previously, the Wingless pathway is essential to control midgut stem cell proliferation. ECs are responsible for activating Wingless signaling in the midgut. When Wingless signaling decreases due to Axin elevation in ECs, midgut stem cells undergo aberrant proliferation under homeostatic conditions.

\textit{Apc1}, \textit{pygo} (Pygorus-Wingless transcription co-factor) and \textit{arm} (Armadillo-Wingless transcription factor, \( \beta \)-catenin in mammals) are indispensable for Wingless-dependent regulation of stem cell proliferation in the adult midgut. Therefore, the genetic depletions of them result in an increased number of esgcargot-LacZ positive (\( esg^{+} \)) midgut stem and progenitor cells around RFP negative mutant clones compared to FRT-RFP control clones (Fig 3.10A-D).

\textbf{3.3.5 Unlike \textit{Tnks}-null mutants, \( Tnks^{K598A} \) knock-in mutation does not promote proliferation of ISCs in \textit{Drosophila}}

\( TNKS \) is a positive regulator of Wingless signaling. I found that \( Tnks \)-null mutant clones had an increased number of Arm\( ^{+} \)/Pros\( ^{+} \)midgut stem cells (Fig 3.11A) as well as \( esg^{GFP^{+}} \) stem and progenitor cells in \textit{Drosophila} midguts (Fig 3.11B). \( Tnks \) depletion caused accumulation of mis-expressed GFP-Axin in ECs (Fig 3.12A). Loss-of \( Tnks \) non-autonomously promoted stem cell division; as a consequence, there were an increased number of \( esg^{GFP^{+}} \)
Figure 3.13 Pharmacological inhibition of Axin degradation suppresses Wingless signaling.

A- XAV939 and Bortezomib treatment inhibited Axin degradation in Drosophila midguts. 2-day-old female FRT82B, Axn<sup>h</sup>, BAC-Axin-V5 flies were treated with Bortezomib or XAV939. DMSO was also supplemented in the low melt fly food as a control of drug treatments. Axn<sup>h</sup> is a loss-of function mutant of Axin. This mutation leads to lethality in Drosophila. BAC-Axin-V5 transgenic line rescues the lethality of Axn<sup>h</sup> mutant. After 5-day XAV939 or Bortezomib treatment, midguts were dissected for V5-immunostaining. Axin accumulation was observed in dissected midguts. B- XAV939 treatment caused Axin accumulation. After 5-day XAV939 or Bortezomib treatment, total proteins were extracted from these flies and analyzed by V5-immunoblotting. ‘‘se’’ meant short exposure of immunoblotting while ‘‘le’’ meant long exposure. C- Quantification of V5-Axin immunoblotting upon XAV939 treatment. D- Bortezomib treatment resulted in Axin elevation. E- Quantification of V5-Axin immunoblotting after Bortezomib treatment. F- Bortezomib inhibited Wingless signaling. fz3>GFP reporter activity mainly reduced in the posterior midgut. 5-day Bortezomib-fed female flies were dissected, and fz3>GFP-expression in posterior midguts was examined with confocal microscope.
**Figure 3.13**

**A**

![Image of immunofluorescence staining for V5-Axin in DMSO, XAV939, and Bortezomib samples.](image)

*BAC-AxinV5, FRT82B, Axin^e*

**B**

![Image of Western blot analysis for V5-Axin with DMSO and XAV939.](image)

V5-Axin, WB: α-V5 (se)

V5-Axin, WB: α-V5 (le)

β-Actin

**C**

![Graph showing relative V5-Axin levels for DMSO and XAV939.](image)

**D**

![Image of Western blot analysis for V5-Axin with DMSO and Bortezomib.](image)

V5-Axin, WB: α-V5 (se)

V5-Axin, WB: α-V5 (le)

β-Actin

**E**

![Graph showing relative V5-Axin levels for DMSO and Bortezomib.](image)

**F**

![Image of immunofluorescence of frizzled3-GFP in DMSO and Bortezomib samples.](image)

*Drosophila midgut-hindgut boundary*
Figure 3.14 XAV939 and Bortezomib induce JAK-STAT signaling to promote ISC proliferation. A- XAV939 and Bortezomib treatments drove stem cell hyper-proliferation in adult midguts compared to DMSO-fed flies. B- XAV939 and Bortezomib triggered JAK-STAT signaling, traced with a 10xStat-GFP reporter. C- XAV939 and Bortezomib induced upd3>GFP expression. D- XAV939 or Bortezomib increased stem cell proliferation in adult midguts. 2-day-old Oregon R female flies were fed with 50µM XAV939 or Bortezomib for 5 days. Midguts were then dissected and stained with α-pH3 antibody. pH3+ mitotic cells were quantified in each midgut analyzed. Posterior midguts were imaged by confocal microscope. n>10. Each dot represented an animal and lines indicated means ± s.d. ***p< 0.0001. Statistical analyses were done with One-way ANOVA. Scale bar: 10µm.
Figure 3.15 Bortezomib-mediated ISC proliferation requires JAK-STAT activation. A- DMSO treatment did not affect stem cell division in midguts. B- Bortezomib treatment led to over-proliferation of midgut stem cells. C- Depletion of the Stat92E transcription factor suppressed Bortezomib-induced stem cell division. D- Inactivation of upd2/3 reversed the proliferation effect of Bortezomib on stem cells. E- The reduction of JAK-STAT signaling in ISCs by the expression of UAS-Socs36E reduced the proliferation response to Bortezomib. A UAS-Socs3E transgene expression was driven by esg-Gal4>GFP. 2-day-old Oregon R female flies were fed with 50µM XAV939 or Bortezomib for 5 days. These flies had esg-Gal4>GFP in their backgrounds. Posterior midguts were imaged by confocal microscope. n>5 for each experimental condition.
Figure 3.16 Tnks$^{K598A}$ knock-in mutation does not affect stem cell proliferation in the adult midgut. A- Tnks$^{503}$-null flies have more Arm$^+/$/Pros$^-$ midgut stem cells. B- Unlike Tnks-null flies, Tnks$^{K598A}$ mutation did not affect Arm$^+/$/Pros$^-$ stem cell numbers. Tnks$^{K598A}$ did not caused any changes in the JAK-STAT pathway. A 10xStat-GFP reporter fly line was used to monitor JAK-STAT signaling. C- The quantification of Arm$^+/$/Pro$^-$ ISCs in midguts of Tnks-null (Tnks503) flies. D- The quantification of Arm$^+/$/Pro$^-$ ISCs in midguts of Tnks$^{K598A/+}$ flies. Tnks$^{K598A/+}$ flies were used as control flies. E- Tnks$^{K598A}$ flies did not affect upd3>GFP expression. Tnks$^{K598A/+}$ flies were used as control flies. F- The quantification of pH3$^+$ mitotic cells. There were no differences in the average of pH3$^+$ cell numbers between heterozygous or homozygous Tnks$^{K598A}$ flies. Oregon R flies were used as wild type flies. Scale bar: 50µm. 7-day-old female flies were dissected, and posterior midguts were examined with confocal microscope. Each dot represented an animal. Graphs indicated means ± s.d. Statistical analyses were done with two-tailed Student’s t-test. ns means not significant. Scale bars: 10µm for A, C and 50µm for E.
Figure 3.16

A

$Tnks^{503/+}$

$Tnks^{503/503}$

Arm/Pros/DAPI

B

$Tnks^{K598A/+}$

$Tnks^{K598A/K598A}$

Stat-GFP/Arm/Pros/DAPI

C

$p<0.0079$

Y-axis: Arm/Pros ISCs / field

D

Y-axis: Arm/Pros ISCs / field

E

$Tnks^{K598A/+}$

$Tnks^{K598A/K598A}$

udp32-GFP/DAPI

F

Y-axis: pH3+ ISCs / PMG

ns
stem and progenitor cells in adult midguts (Fig 3.12B-E). Ectopic expression of Flag-tagged TNKS in ECs suppressed over-proliferation of ISCs in Tnks-null mutants (Fig 3.12D-E).

Both pharmacological inhibition of Axin ADP-ribosylation by XAV939, and its 26S proteasome-dependent degradation by Bortezomib, led to an accumulation of endogenous Axin in Drosophila midguts (Fig 3.13A). I also detected increased levels of endogenous Axin in the whole-body protein lysates from XAV939 or Bortezomib fed flies (Fig 3.13B-E). High levels of Axin suppressed the expression of frizzled3, an indicator of Wingless signaling activity, in the midgut. Therefore, I observed more esg\textsuperscript{+/GFP} stem and progenitor cells after XAV939 or Bortezomib treatment (Fig 3.14A). Similar to Tnks-null mutants, XAV939 and Bortezomib treatments both triggered the JAK-STAT pathway (Fig 3.14B) and stem cell proliferation (Fig 3.14D) in Drosophila midguts.

In response to stress, ECs express and secrete UPD cytokines to activate JAK-STAT signaling, which is essential for ISC to proliferate. I, therefore, used upd3\textsuperscript{Gal4} enhancer trap to monitor upd3 induction in midguts and found that upd3 expression correlated with the effects of XAV939 or Bortezomib treatment (Fig 3.14C).

When I reduced JAK-STAT signaling in esg\textsuperscript{+/GFP} cells, either by inactivation of transcription factor Stat9E and upd2-3 cytokines or by over-expression of Socs36E-the inhibitor of the JAK-STAT pathway, Bortezomib treatment-induced hyper-proliferation response was suppressed (Fig 3.15).

I next analyzed 7-day-old female Tnks-null as well as Tnks\textsuperscript{K598A} mutants. I only found an increase in the numbers of Arm\textsuperscript{+/Pro}\textsuperscript{-} stem cells in Tnks-null midguts compared to control flies (Fig 3.16A, C). However, Tnks\textsuperscript{K598A} mutant did not promote the numbers of Arm\textsuperscript{+/Pro}\textsuperscript{-} stem cells in midguts (Fig 3.16B). I did not indicate any significant change in the number of stem cells
(Fig 3.16D) and pH3+ mitoticISCs in Tnks$^{K598A}$ flies (Fig 3.16F). Furthermore, both Stat-GFP reporter activity (Fig 3.16D) and upd3>GFP gene expression were not induced by Tnks$^{K598A}$ (Fig 3.16E). Taken together, K598 does not involve in the ADP-ribosylation of Axin as well as its degradation in vivo.

I finally tested the effect of K598 residue on TNKS degradation in S2R+ cells. Unlike PARP-deficient mutants, K598A mutant still underwent proteolytic degradation in the presence of Iduna in S2R+ cells (Fig 3.17A-B). XAV939 stabilized both K598A and wild type TNKS (Fig 3.17B). Flag-tagged wild type or K598A TNKS was co-expressed with Iduna in S2R+ cells to see the PARsylation levels of TNKS. An equal amount of total proteins was pulled down via Flag beads and then immunoblotted with a PAR antibody. While there was less ADP-ribosylation in K598A compared to wild type TNKS, it was still adequate for its degradation (Fig 3.17B). Therefore, I suggest that K598 residue in the ARC4 domain may be essential for other physiological function(s) instead of Axin and TNKS ADP-ribosylation and their degradations.

### 3.3.6 Lysine 598 is essential for TNKS-binding to certain proteins

Tnks$^{K598A}$ knock-in mutants have several phenotypes that overlapped with Tnks-null mutants. To investigate the most affected molecular target(s) of K598, I conducted Co-IP-MS analysis (Fig 3.18A). I expressed Flag-tagged wild type (Fig 3.19A) or K598A TNKS (Fig 3.19B) in S2R+ cells and pulled down Flag-tagged TNKS to reveal which binding partner(s) were significantly reduced for K598A TNKS (Fig 3.18B). We then compared wild type TNKS interactome with the interactome of K598A (Fig 3.18B-C). As a result, we identified 11 proteins as the most prominently affected binding partners of TNKS.
Figure 3.17 K598A TNKS is auto-poly-ADP-ribosylated and degraded in S2R+ cells. A- Iduna degraded K598A mutant in S2R+ cells, whereas PARP-deficient mutants were resistant against Iduna-dependent TNKS degradation. B- XAV939 treatment inhibited Iduna-mediated destruction of K598A mutant protein. TNKS wild type or the mutants were expressed in S2R+ cells. 20μM XAV939 and DMSO treatments were done 24h after transfection. Following 48 h of XAV939 treatment, S2R+ cells were collected and lysed for Western blotting against Flag-TNKS. β-actin was used as a loading control. PARsylated TNKS was detected with a PAR antibody after Flag-TNKS immunoprecipitation (IP).
Figure 3.18 IP-MS identifies 11 proteins as the most prominently affected binding partners of TNKS. A- MS-Based approach to identify the binding partners of *Drosophila* TNKS. B- Flag-peptide-eluted proteins were analyzed with silver staining before MS analysis. C- K598A mutation significantly reduces the binding of several proteins. S2R+ cells expressing wild type Flag-TNKS or K598A Flag-TNKS were collected for an anti-Flag Co-IP. Shown here is a volcano plot of label-free quantitative MS results. Biological triplicates were conducted and analyzed. The cut-off line for significance is P-value<0.001 (-log10P >3) and fold difference log2<-1. Statistically significant proteins were labeled in red. D- TNKS K598A mutation prominently reduces binding affinity of 11-proteins, identified by IP-MS. Statistically significant proteins are listed in this table. Given *p*-values represent the statistical significance, calculated from the three-independent pull-down analyses. Binding abilities of each protein to K598A TNKS are compared to the interactions with the wild type TNKS protein. Proteins name and their possible functions are searched in the Uniprot’s *Drosophila* proteome.
Figure 3.18

A
Actin5C Gal4>Flag-TNKS
Actin5C Gal4
Flag peptide elution
S2R+ cell harvest Flag-immunoprecipitation

B
Control WT-Flag-TNKS K66A-Flag-TNKS Control WT-Flag-TNKS K66A-Flag-TNKS

kDa
170 160 150 140 130

Input Immunoprecipitation

C

D

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Figure 3.19 MS-based approach to identify wild type *Drosophila* TNKS and K598A TNKS interactomes. A- Wild type GKYDICK peptide B- GAYDICK peptides were detected by MS. GKYDICK peptide is in wild type TNKS, whereas GAYDICK is in K598A TNKS.
### Figure 3.19

#### A

Wild type Tankyrase

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#### B

K598A Tankyrase

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These proteins are RH07106p Hsp70 binding protein, Caper, Snapin, Receptor-mediated endocytosis protein 6 homolog, Prx5, RnpS1, IP12463p putative cADPR synthase, Exu, A1Z6G6, U2af50, Pst (Fig 3.18C). These proteins have various suggested functions (3.18D). As a result, I suggested that K598 promotes the formation of complexes between TNKS and these proteins. It, therefore, points out a critical function of K598 in various biological processes. In the future, it will be interesting to investigate the functional consequences of these interactions.
3.4 Material and Methods

Fly stocks: Flies were kept at a 12-hour light/dark cycle. All crosses were performed at 22-25°C unless stated otherwise. The following fly stocks were used for this study (Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC) number (#) given in parentheses):

The stocks used in here: $Tnks^{K598A}$ (generated in this study), $esg$-Gal4, $UAS$-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), $esg$K606 (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), $10XStat$-GFP (Bach et al., 2007), $BAC$-$Axin$V5 (a gift of Jean-Paul Vincent, Gerlach et al., 2014), $FRT$ 82B, $Axhr^b$, $BAC$-$Axin$V5 (a gift of Jean-Paul Vincent, Gerlach et al., 2014), $UAS$-GFP-$Axin$ (BDSC# 7224), $Myo1A$-Gal4, $tub$-Gal80ts, $UAS$-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), $upd3$-Gal4, $UAS$-GFP (a gift of Dr. Norbert Perrimon; Markstein et al., 2014), $Audp2/3$ (BDSC# 129), $ADome$ (BDSC# 12030), $Tnks^{503}$ (a gift of Dr. Yahsi Ahmed, Wang et al., 2016), $Tnks^{19}$ (a gift of Dr. Yahsi Ahmed, Wang et al., 2016), $UAS$-$Tnks$-HA (a gift of Dr. Yahsi Ahmed, Wang et al., 2016), $UAS$-$CG8786/dlduna$ RNAi#1 (BDSC# 40882), $UAS$-$CG8786/dlduna$ RNAi#2 (VDRC#43533), $UAS$-$CG8786/dlduna$ RNAi#3 (VDRC#36028), $UAS$-$CG8786/dlduna$ RNAi#4 (VDRC#36029), and white RNAi (BDSC#33623), $fz3$-Gal4 (BDSC#36520). The rest of Drosophila lines, which were studied here, were obtained from Steller Lab stocks. Oregon R flies were used as control and only adult female flies were analyzed in this study.

Drosophila egg collection: Please refer to Chapter 2, Material and methods.

Fly Climbing: Newly eclosed male or female flies were separated and raised on standard food for 3 days or 1 month. Cohorts of 60 flies were pre-divided into 3 groups and transferred, without anesthesia, into graduated vials. The vials were gently tapped to force the flies down to
the bottom. The climbing behavior was observed for 18 s and repeated 3 times with 2-min intervals.

**Low-melt fly food:** This fly food was developed by Markstein et al., 2014. They developed this fly food formula for mixing drugs in low volumes. It contains low-melt agarose and standard agarose in place of agar. Low-melt fly food was prepared with distilled water containing 2% (wt/vol) autoclaved yeast, 7% (vol/vol) corn syrup, and 1.5% (wt/vol) agarose (composed of 1 part standard agarose to 11 parts of low-melt agarose). Following its sterilization, the food was stored at 4°C, boiled and mixed as liquid with drugs at 37°C. The resulting food-plus drug mixtures solidified at 30°C into soft fly-edible gels.

**CRISPR/Cas9 genome editing for *Tnks*^K598A^**: CRISPR optimal target finder website (tools.flycrispr.molbio.wisc.edu/targetFinder) was used to identify an appropriate guide RNA (gRNA) target sequence within TNKS-ARC4 domain (Gratz et al., 2013, 2014). I found GCAAATATCATACTTTCCCT TGG sgRNA to target lysine 598 in TNKS. By using the forward 5`-GTCGCAAAATATCATACTTTCCCT-3` and reverse 5`-AAACAGGGAAAGTATGATATTG-3` oligos (IDT, Inc.). I cloned the sgRNA into pCFD3-dU6:3-gRNA plasmid (Addgene, plasmid# 49410, Port et al., 2014). Transformants were verified via Sanger sequencing (Genewiz, Inc.). I purchased single strand DNA as a HDR donor sequence for K598A mutation- CTATGAGGTAAACGACTTG AGT AAG CAC GGA GCC AAT GTA AAT GTA TCG GAT TTG TGG AAG TTT ACT CCT CTT CAT GAA GCT GCa GCa AAG GGA gGc TAT GAT ATT TGC AAG CTG CTC TTG AAA CAT GGC GCT GAT CCA ATG AAG AAG AAT CGG GAT GGC GCG ACA CCA (IDT, Inc). The gRNA plasmid and the HDR donor sequence were injected into 300 embryos of Act5C-Cas9
Drosophila (BestGene, Inc.). The injection was yielded 95 G₀ progeny, and I established 125 individual fly lines, a couple of which could possibly have the Tnks<sup>K598A</sup> mutations.

**Isolation of the Tnks<sup>K598A</sup> mutants:** Total DNA was isolated from 5-day-old adults of established K598A lines. To screen the mutant lines, PCR fragments were amplified with specific primers (forward primer 5’-F: CCCTTGAGGGACTTACGGCCGC-3’, reverse primer 5’-CTTGTCCTGTGCATTAACATCGGCTC-3’). Amplified PCR products were then digested with Styl restriction enzyme, which gave two DNA fragments. PAM sequence mutation on sgRNA changed the restriction digestion site. Therefore, Styl did not digest the PCR product. Based on Styl-RFLP, I identified more than 10 fly lines. To verify the mutation, amplified PCR products were sent for DNA sequencing (Genewiz, Inc.).

**Clone analyses and RNAi experiments:** Please refer to Chapter 2, Material and methods.

**Cell culture:** Please refer to Chapter 2, Material and methods.

**Western blot analyses:** Please refer to Chapter 2, Material and methods.

Primary antibodies: anti-Flag-HRP (1/1000, Sigma-Aldrich, A8592), anti-Flag (1/1000, CST, D6W5B 14793), anti-PAR (1/1000, Trevigen, 4335-MC-100), anti-Myc tag (1/1000, CST, 9B11, 2276), anti-Axin Drosophila (Santa Cruz, dT20, sc15685), anti-tubulin DM1A clone (1/1000, Sigma-Aldrich, T9026), mouse anti-GFP-HRP (1/2500, clone B2, Santa Cruz Biotechnology, sc-9996-HRP), rabbit anti-β-Actin-HRP (1/5000, clone 13E5, Cell Signaling Technology, 5125), donkey anti-rabbit-HRP (1/5000, Jackson ImmunoResearch, 711-035-152), donkey anti-mouse-HRP (1/5000, Jackson ImmunoResearch, 715-035-150), donkey anti-guinea pig-HRP (1:5000, Jackson ImmunoResearch, 706 006 148).

**Immunofluorescence:** Please refer to Chapter 2, Material and methods.
Primary antibodies: mouse anti-Arm (Wang et al., 2016; N2 7A1, DSHB, 1:50), mouse anti-Propero (Wang et al., 2016; MR1A, DSHB, 1/50), mouse anti-GFP (GFP-12A6, DSHB, 1/100), mouse anti-β-galactosidase (Tian et al., 2016); 401A, DSHB, 1/100), mouse anti-Delta (Wang et al., 2016; C594.9B, DSHB, 1/100), rabbit anti-phosho-S10-Histone3 (Wang et al., 2016; 06-570, Millipore, 1/1000). The secondary antibodies were goat anti-mouse-Alexa 488 plus (Thermo Fisher Scientific, A32723), goat anti-mouse-Alexa 568 (Thermo Fisher Scientific, A11031), goat rabbit-Alexa 546 (Thermo Fisher Scientific, A11035), goat rabbit-Alexa 488 (Thermo Fisher Scientific, A11034), goat anti-rabbit-Alexa 633 (Thermo Fisher Scientific, A21071) and used at 1/1000.

**Immunoprecipitation:** Please refer to Chapter 2, Material and methods.

**Recombinant protein purification from S2R+ cells:** Please refer to Chapter 2, Material and methods.

**Expression and Purification of of human NUDT16:** 1.5 ml of transformed Rosetta2 (DE) cells were grown in terrific broth (TB) medium in the presence of 100µg/ml kanamycin and 34µg/ml chloramphenicol at 37°C. When the absorbance (A) reached 2.0, the temperature was decreased to 18°C. When the A reached 3.0, the expression of recombinant protein was induced with 0.2mM IPTG and continued overnight. Bacterial pellet was prepared by centrifuge at 5000 rpm for 5 minutes. The pellet were then re-suspended in a buffer composed by 20mM Tris/HCl, pH 8.0, 150mM NaCl, 10mM imidazole, 5µM 2-mercaptoethanol, 10% glycerol and supplemented with Complete Protease Inhibitor (Roche). Re-suspended bacterial pellet was then sonicated in order to lyse bacterial cells. After 10-15 min ice incubation, the lysate was clarified by centrifugation and supernatant applied on 5ml of Cobalt beads (GE Healthcare) for affinity purification. The histidine-tagged hNUDT16 was eluted in 300mM imidazole. Finally, the
elution buffer of the protein was exchanged with a buffer composed of 25mM Tris, pH 8.0, 300mM NaCl, 10% glycerol and 1mM DTT. Proteins were then concentrated to 10mg/ml using 10 kDa Amicon-Ultra centrifugal filter (Millipore).

**In vitro ADP-ribosylation:** 20 mgs of Flag-tagged *Drosophila* TNKS protein (purified from S2R+ cells) were automodified in 100 µL of a reaction buffer containing 100mM Tris-HCl pH 8.0, 10mM MgCl2, 10mM DTT, 25µM NAD+ for 1h at 24-25°C.

**hNUDT16 digestion:** After the auto-modification reaction of TNKS, 5 µL of purified hNUDT16 was added to digest TNKS-bound pADPr (3h at 30 °C). The MgCl2 (Sigma) concentration was adjusted to 15mM to allow full Nudix hydrolase activity. Auto-modified TNKS was then incubated for 3h at 30°C with hydrolytic enzymes in 10 µl of reaction mixture.

**Mass spectrophotometry for ADP-ribose acceptor side identification:** Flag-TNKS was purified from S2R+ cells and 60mg ADP-ribosylated *in vitro*. After ADP-ribosylation, it was incubated with hNUDT16 for 3h at 30°C. After enzymatic reactions, proteins were precipitated by cold acetone. Pellets were dissolved in 6M guanidium-chloride (Sigma), reduced with TCEP (Pierce) and alkylated with chloroacetamide (Sigma). After a 10-fold dilution with 50mM ammonium bicarbonate (Fluka), proteins were digested using 200ng of trypsin Gold (Promega) for 16 h at 37°C. Samples were then desalted. Later, dried samples were reconstituted in 5 µL of 0.1% trifluoroacetic acid (TFA) and 25 µL of 6%TFA/80%ACN. Twenty microliters of a 10mg/mL water solution of TiO2 (ZirChrom Separations, Inc.) beads was added to a homemade gel-loading tip that contained a glass fiber frit. Gentle air pressure supplied from a syringe was applied to speed the flow of solvent and beads were always left wet. Ten microliters of 6%TFA/80%ACN was used to wash the beads. Sample was passed through the column three times and the final elute was collected. Two wash steps were each performed twice: first with 10
µL of 6%TFA/80% ACN followed by 10 µL of 0.1% TFA. Each wash elute was collected. Ten microliters of 5% ammonium hydroxide was used to elute the capture peptides. Sixty microliters of 2% FA was used to immediately adjust the final elution pH. All samples were dried to near completion and reconstituted in 5% ACN/ 0.1% FA for mass spectrometry analysis. MS was performed by The Rockefeller University Proteomics core facility.

**Co-IP Mass spectrophotometry to identify TNKS-binding partners:** S2R+ cells were transfected with *Act5C-Gal4* and *UAS-Flag-Tnks* or *UAS-Flag-K598A-Tnks*. After 72h, S2R+ cells were collected and *Act5C-Gal4* expressing S2R+ cells were used as a negative control in IP experiment. Both Flag-tagged TNKS and *Act5C-Gal4* expressing cells were lysed and immunoprecipitated with Flag beads in lysing buffer. Finally, IP samples (3 from control and 3 from Flag-TNKS expression) were eluted with Flag-peptide. Eluted proteins were cleaned up by using SDS-PAGE, reduced (10mM DTT) and alkylated (30mM iodoacetamide), followed by digestion with a cocktail of LysC and Trypsin Gold. Digestions were halted by adding trifluoroacetic acid (TFA) and digests then were desalted and analyzed by reversed phase nano-LC-MS/MS using either a Fusion Lumos or a Q-Exactive Plus (Thermo Fisher Scientific) both operated in high/high mode. Data were searched and quantified against Uniprot’s *Drosophila* proteome databases using ProteomeDiscoverer v. 1.4.0.288 (Thermo Scientific) combined with Mascot v. 2.5.1 (Matrix Science) and/or MaxQuant v. 1.6.0.13. Oxidation of methionine and protein N-terminal acetylation were allowed as variable modifications and all cysteines were treated as being carbamidomethylated. Peptide matches were filtered using a Percolator-calculated false discovery rate (FDR) of 5%.

**Statistics:** Student *t*-test and ANOVA were used as statistical analysis and those were done with Prism (GraphPad) software.
Acknowledgment: Dr. Milica Tesic Mark from The Rockefeller University proteomics core facility, conducted MS analyses for ADP-ribose acceptor side identification as well as TNKS-interactome.
4 ADP-ribosylation by TNKS provides a molecular scaffold for rapid protein degradation

4.1 Summary

ADP-ribosylation affects the fate of proteins by directly modulating their activity or stability. By and large, TNKS-mediated poly-ADP-ribosylation is tightly coupled to the 26S proteasome-mediated proteolysis. Auto-poly-ADP-ribosylation is an inhibitory post-translational modification for TNKS as well. Here, I find that the ubcD1 E2 ubiquitin-conjugating enzyme (an E2 ubiquitin ligase) participates in Iduna-dependent TNKS degradation by binding to a poly-ADP-ribose scaffold. Strikingly, adding low concentration of poly-ADP-ribose polymers provides a signal to stimulate auto-ubiquitylation of Iduna in the presence of ubcD1. Similarly, ubcD1 promotes Iduna-dependent ubiquitylation of PARsylated TNKS \textit{in vitro}. \textit{ubcD1}-RNAi results in elevation of Iduna, Tankyrase, and Axin proteins in \textit{Drosophila}. These results demonstrate that ubcD1 mediates Iduna-dependent degradation of certain proteins. Finally, TNKS can colocalize with active 26S proteasomes in the \textit{Drosophila} midgut. Inhibition of PARP activity by XAV939 suppresses this co-localization. As a result, I suggest that poly-ADP-ribosylation may play a role as a molecular scaffold that brings together TNKS, ubcD1 E2 ubiquitin-conjugating enzyme, Iduna E3 ubiquitin ligase, and the substrates to close proximity for rapid degradation of target proteins.
4.2 Introduction

As one of the poly-ADP-ribose polymerases (PARPs), TNKS consumes NAD+ to synthesize poly-ADP-ribose (PAR). Although TNKS-mediated poly-ADP-ribosylation (PARylation) is largely associated with the 26S proteasome-mediated protein degradation, it can also promote the activity of several proteins. TNKS-mediated PARylation of PI31 and JNK enhances proteasome assembly and signal transduction, respectively rather than destruction of PI31 and JNK in *Drosophila* (Park and Steller, 2013; Li et al., 2019). Furthermore, several TNKS-interacting proteins do not undergo PARylation. GDP-mannose-4, 6-dehydratase is a cytoplasmic TNKS1 binding-protein that inhibits PARP activity of TNKS (Bisht et al., 2012).

PAR is a negatively charged biopolymer that has an impact on protein stability as well as degradation. TNKS can reside in multiple subcellular localizations and participate in various biological processes including telomere homeostasis, and Wnt/β-catenin signaling. In 3T3-L1 adipocytes, TNKS localizes in GLUT4 vesicles near the Golgi (Chi and Lodish, 2000). PARylation can also recruit PAR-binding proteins into a close proximity. PAR-binding proteins, ``PAR readers``, recognize PAR polymers through PAR-binding motif (PBM). Following the PAR reading, certain proteins co-localize on the PAR scaffold to assemble protein complexes during stress granule formation, DNA repair and ribosomal RNA maturation (McGurk et al., 2018; Duan et al., 2018; Boamah et al., 2012; Caldecott, 2014; Fatokun et al., 2014; Hotiger et al., 2015; Hsiao and Smith, 2008; Krietsch et al., 2013; Teloni and Altmeyer, 2016; Catara et al., 2017; Isabella et al., 2012; Leung et al., 2011). For instance, nuclear PARP1 facilitates ribosomal RNA (r-RNA) biogenesis in *Drosophila*. PARP1 becomes automodified upon each act of transcription start within r-RNA gene. Auto-PARsylated PARP1 serves as a chaperoning machine during the whole cycle of ribosome maturation in the nucleus. The
dynamic PARP1-mediated PAR scaffold brings a subset of nucleolar proteins to the proximity of precursor r-RNA and coordinates the order of events including r-RNA processing, modification and loading of subsets of ribosomal proteins to mature pre-ribosome in Drosophila (Boamah et al., 2012). Depletion of Parp leads to removal of PAR-binding proteins from nucleoli, which disrupt processing, modification, and folding of ribosomal RNA in Drosophila (Boamah et al., 2012).

In this chapter, I demonstrated that ubcD1 E2 ubiquitin ligase is involved in Iduna-dependent TNKS degradation. Loss-of ubcD1 resulted in an elevation of mis-expressed Iduna, and TNKS as well as endogenous Axin in Drosophila. I found that the E2 ubiquitin ligase ubcD1 promotes auto-ubiquitylation of Iduna in the presence of PAR. However, ubcD1 does not interact with Iduna or TNKS, ubcD1 plays a role in Iduna-dependent TNKS ubiquitinylation. Moreover, ubcD1 is capable of binding ADP-ribose scaffold because of its PAR-binding motifs. I finally showed that TNKS co-localizes with active 26S proteasomes in the Drosophila midgut. Taken together, I provided evidence that ADP ribosylation by TNKS brings ubcD1, Iduna, and the target protein into close proximity for accelerating degradation of the target protein.
4.3 Results

4.3.1 Proteomic screen to identify TNKS-binding partners for the regulation of its stability

TNKS-mediated PARsylation is mainly related to UPS-mediated protein destruction. PARsylation destabilized both TNKS itself and Axin by recruiting a PAR-directed E3 ubiquitin ligase Iduna in Drosophila (Gultekin and Steller, 2019). Thanks to PAR reader proteins, ADP-ribosylation can mediate the assembly of protein complexes. Therefore, I suggest that ADP-ribosylation provided by TNKS may provide a molecular scaffold for rapid degradation of TNKS itself or its substrates. To this end, I conducted a Co-IP-MS of Flag-tagged TNKS to identify its binding partners.

The Drosophila TNKS interactome was then narrowed down to the proteins, related to the Ubiquitin-Proteasome system since they might regulate TNKS degradation. I found that the E2 ubiquitin-conjugating enzymes Ben and Morgue, E3 ubiquitin ligase Iduna, deubiquitinating enzymes (DUBs) Usp30 and Usp10, and finally 19S proteasome subunits Rpt1, 2, 3, and 6 were significantly co-immunoprecipitated with Flag-tagged TNKS in S2R+ cells (Table 4.1). I assessed an RNAi-based validation screen to ask if these binding-partners might be involved in TNKS degradation. HA-tagged TNKS-expressing transgenic flies were crossed with RNAi lines under the temperature sensitive Tub-Gal4 driver. HA-tagged TNKS was then detected via immunoblotting from whole-body protein lysate. A screen for compatible E2 ubiquitin conjugating enzymes in an in vitro RNF146 auto-ubiquitinylation assay reveals UbcH5a as a functional partner for human Iduna (Zhang et al., 2011). Drosophila ubcD1 is ortholog of the human UbcH5a gene. Although, Flag-TNKS did not pull down ubcD1 in my IP-MS, I included ubcD1 gene into the RNAi screen (Fig 4.2A).
Table 4.1 *Drosophila* TNKS interactome that can be related to TNKS ubiquitinylation and degradation. *Drosophila* Tankyrase interactome is narrowed down to the proteins that can be related to TNKS degradation. Flag-tagged TNKS was expressed under Act5C-Gal4 driver in S2R+ cells and pulled down with Flag beads.

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**Figure 4.1 ubcD1 plays a role in TNKS degradation.** A- Both Usp30 and Usp10 have roles in the regulation of TNKS protein stability. *Usp10* depletion by RNAi resulted in TNKS elevation. Decrease in the *Usp30* gene dosage by half resulted in reduction in the protein level of TNKS. B- RNAi-mediated *ubcD1* inactivation led to an elevation of HA-tagged TNKS protein. *UAS-HA-Tnks* transgene was expressed under the temperature sensitive *Tub-Gal4* driver. 7-day-old adult females were lysed. Western blotting was done with an anti-HA antibody. C- *ubcD1* knockdown also resulted in an accumulation of PARsylated TNKS. Western blot was performed with an anti-PAR antibody. a-Tubulin was a loading control. D- *ubcD1* deficient larvae had more HA-tagged TNKS. 3rd instar larvae were dissected and midguts were stained against HA and Armadillo. E- *ubcD1* knockdown resulted in elevation of HA-tagged TNKS as well as PAR polymers in adult midguts. 7-day-old adult females were dissected. Dissected midguts were stained against HA and PAR.
I found that Usp10 depletion by RNAi resulted in TNKS elevation. In contrast, reducing the Usp30 gene dosage by half led to decrease in the protein level of TNKS (Fig 4.1A). These results suggested that both both Usp30 and Usp10 have roles in the regulation of TNKS protein stability. On the other hand, morgue-RNAi did not cause any change in the protein level of TNKS. Since Morgue has a putative TNKS-binding motif, RNAi result can suggest that Morgue ubiquitin-conjugating enzyme may work together with TNKS to regulate protein stability of one of the target proteins of TNKS. However, RNAi-mediated ubcD1 inactivation led to an elevation of HA-tagged TNKS (Fig 4.1B). ubcD1 knockdown also resulted in an accumulation of PARsylated TNKS (Fig 4.1C). Finally, both larval (Fig 4.1D) and adult midguts (Fig 4.1E) had more HA-tagged TNKS as well as PAR polymers when ubcD1 was depleted (Fig 4.1E).

4.3.2 ubcD1 promotes TNKS degradation

ubcD1 also known as effete, encodes a 147-amino acid ubiquitin-conjugating enzyme that is similar to the mammalian ubcH5 class (Fig 4.2A). As a result of amino acid sequence alignment, ubcD1 is conserved from flies to human (Fig 4.2B). I next assessed if ubcD1 genetically interacted with Iduna, by crossing ubcD1-RNAi flies to Myo1A\textsuperscript{ts} > UAS-GFP-Myc-Iduna. I found that inactivation of ubcD1 resulted in an elevation of Iduna protein (Fig 4.3A-B). Hence, ubcD1 promotes ubiquitinylation activity of Iduna in Drosophila.

By and large, ubiquitin ligases physically interact with their ubiquitin-conjugating enzymes to transfer ubiquitin to their substrates. To test if ubcD1 protein interacted with Iduna, I carried out a Co-IP assay. ubcD1 and Iduna co-expressing flies were lysed to pull down AcGFP-Iduna. Surprisingly, Iduna did not co-immunoprecipitate with ubcD1 in Drosophila (Fig 4.3C).
Figure 4.2 ubcD1 is conserved from flies to human. A- ubcD1/effete encodes a 147-amino acid ubiquitin-conjugating enzyme that a member of mammalian ubiquitin-conjugating ubcH5 class. Hs, Homo sapiens; Mm, Mus musculus; Bt, Bos taurus; Dm, Drosophila melanogaster. Amino acid sequences were aligned and phylogenetic tree was drawn with Clustal Omega software. B- ubcD1 is conserved from flies to human.
Figure 4.3 ubcD1 regulates the ubiquitin ligase activity of Iduna. A- ubcD1 knockdown elevated AcGFP-Iduna in Drosophila midguts. B- The quantification of AcGFP-Iduna protein levels upon ubcD1-RNAi. UAS-AcGFP-Myc-Iduna transgene was expressed under the temperature sensitive Myo1A-Gal4 driver. 7-day-old adult females were lysed. Western blotting was done with an anti-GFP antibody. C- Iduna did not pull down ubcD1. UAS-AcGFP-Myc-Iduna or UAS-ubcD1-HA or UAS-AcGFP-Myc-Iduna, UAS-ubcD1-HA transgenes were expressed under the temperature sensitive Tub-Gal4 driver. 7-day-old adult females were lysed. Immunoprecipitation was conducted with anti-GFP antibody Western blotting was done with GFP and HA antibodies.
Figure 4.3

A  
Myo1A Gal4b>AcGFP-Ilduna

RNAi:  
170  
130  
100  
55  
40

AcGFP-Ilduna  
β-actin

B  
Relative AcGFP-Ilduna protein level

0  
1  
2  
3

white RNAi  
ubcD1 RNAi

Myo1A Gal4>

C  
Tub Gal4b>

+/

AcGFP-Ilduna  
ubcD1-HA  
AcGFP-Ilduna/ubcD1-HA  
ubcD1-HA  
ubcD1-HA  
ubcD1-HA  
ubcD1-HA

100  
70  
25  
15

AcGFP-Ilduna  
ubcD1-HA

Total lysates  
Co-immunoprecipitates
This result suggests Iduna may not directly bind to ubcD1 and TNKS or PAR modification may be required for ubcD1 and Iduna to interact.

To determine if ubcD1 directly mediated Iduna auto-ubiquitylation, I reconstituted ubiquitylation reaction \textit{in vitro} by using recombinant E1, ubcD1, and Iduna. Ubiquitylation of Iduna was detected by immunoblotting against GST-ubiquitin. In this assay, ubcD1 slightly promoted Iduna auto-ubiquitylation. In the presence of PAR polymers, ubcD1 induced auto-ubiquitylation of Iduna (Fig 4.4A).

Next, PARsylated TNKS was used to test if ubcD1 was required for the substrate ubiquitylation of Iduna. Following TNKS ADP-ribosylation \textit{in vitro}, a ubiquitylation assay was conducted. I found that ubcD1 together with Iduna ubiquitylated PARsylated TNKS (Fig 4.4B). These results demonstrate that ubcD1 participates in Iduna-dependent ubiquitylation and PAR or substrate PARsylation promotes this activity.

**4.3.3 ubcD1 can bind to PAR polymer**

Although TNKS interacts with its protein partners through TBM, ubcD1 does not have a canonical TBM motif. Since ubcD1 did not bind to Iduna directly, I suggested that ubcD1 might bind to the PAR polymer. The most common PAR-binding module is the PBM (Teloni and Altmeyer, 2016). Alignment of the PBM \([HKR]_1-\times_2-\times_3-[AIQVY]_4-[KR]_5-[KR]_6-[AILV]_7-[FILPV]_8\) to ubcD1 revealed that it had two putative PBMs: One PBM is MALKRINK in the amino terminus and the second PBM is LAREWTRKYM found in the carboxyl end of \textit{Drosophila} ubcD1 protein (Fig 4.5A). \textit{UAS-ubcD1-HA} was expressed in the fly midgut. Under denaturating conditions, I observed that ubcD1-HA co-immunoprecipitated with PAR polymers from fly lysates (Fig 4.5B).
Figure 4.4 ubcD1 directly mediates Iduna-dependent ubiquitinylation in the presence of PAR. A- The reconstitution of ubiquitinylation reaction in vitro by using recombinant E1, ubcD1, and Iduna. Auto-ubiquitinylation of Iduna was detected by GST-immunoblotting. B- ubcD1 is involved in Iduna-dependent ubiquitylation of TNKS. Ubiquitylation was reconstituted in vitro using recombinant E1, ubcD1, and Iduna. Flag-tagged TNKS was purified from S2R+ cells and was ADP-ribosylated in vitro. Ubiquitylation was detected by GST-immunoblotting.
**Figure 4.5 ubcD1 binds to PAR polymer through PAR-binding motifs.**

A- *Drosophila* ubcD1 aligned with a canonical PBM that suggested the existence of two PBMs, PBM1-MALKRINK at the amino terminus and PBM2-LAREWTRKYAM at the carboxyl terminus. B- ubcD1-HA co-immunoprecipitated with PARsylated proteins from fly lysates. *UAS-ubcD1-HA* transgene was expressed under *Myo1A-Gal4* driver. 7-day-old adult flies were dissected and midguts were lysed for immunoprecipitation. IP and IB were done with anti-HA and anti-PAR antibodies, respectively. IgG pull down was a negative control. C- ubcD1 bound to PAR. Recombinant GST-tagged ubcD1 was spotted onto a nitrocellulose membrane with a negative control GST and incubated with biotin-PAR polymers. Dot blot was carried with an anti-streptavidin antibody.
**Figure 4.5**

**A**

Canonical PAR binding motif

AVILMFYM$_{1-X_2}$-KR$_3$-X$_4$-AVILMFYW$_5$-AVILMFYW$_6$-KR$_7$-KR$_8$-AVILMFYW$_9$-AVILMFYW$_{10}$

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<td>Dm_ubcD1</td>
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N-terminus PAR motif (Motif 1)

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<tr>
<td>Dm_ubcD1</td>
<td>LAREWTRKYAM</td>
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C-terminus PAR motif (Motif 2)

**B**

IP: IgG HA-ubcD1

WB: α-PAR

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WB: α-HA WCL

**C**

GST GST-ubcD1

IB: α- streptavidin

Ponceau staining

Biotin-PAR dot blot analysis

*Myo1A Gal4>HA-ubcD1*
Figure 4.6 ubcD1 inactivation promotes stem cell division in the Drosophila midgut. A- There were slightly more esg>GFP+ stem and progenitor cells in FRT82B, ubcD1-/- clones compared to FRT82B control clones. 7-day-old adult females were dissected to analyze esg-GFP+ cells. B- ubcD1-RNAi midguts had more Arm+/Pros- stem cells compared to white-RNAi. UAS-RNAi flies crossed with Myo1A-Gal4 lines. 7-day-old females were dissected to analyze stem cell numbers by confocal microscopy. Dissected midguts were stained with anti-Armadillo and Prospero antibodies. Posterior midguts were imaged with a confocal microscope. C- ubcD1-RNAi resulted in elevation of endogenous Axin. D- The quantification of pH3+ -stained cells in midguts. 7-day-old female flies were analyzed by pH3 immunostaining. pH3+ cells were counted in posterior midguts. E- ubcD1-RNAi induced an upd3>GFP reporter compared to white knockdown. 14-day-old posterior midguts were analyzed with a confocal microscope for upd3>GFP. Statistical analyses was done with the Two-tailed Student’s t-test. n>5. ****p>0.0001. Statistics represented as mean ± s.d. Scale bar: 10µm.
To ascertain whether ubcD1 could bind to PAR, I carried out a PAR-binding dot blot. Recombinant GST-tagged ubcD1 was spotted onto a nitrocellulose membrane with a negative control GST and incubated with the biotin-PAR polymer. After immunoblotting with an anti-streptavidin antibody, I found that ubcD1 bound to PAR (Fig 4.5C). These data suggest that ubcD1 is capable of binding ADP-ribose scaffold to mediate Iduna-dependent ubiquitinylation.

I previously showed that both Tnks and Iduna inactivation lead to Axin elevation and stem cell over-proliferation in the Drosophila midgut. To examine the function of ubcD1 in midgut stem cells in vivo, I generated mosaic animals with ubcD1−/− by using Flipase/FRT system. I found there were slightly more esg>GFP+ stem and progenitor cells around FRT82B, ubcD1−/− clones compared to FRT82B control clones (Fig 4.6A). I also demonstrated that ubcD1-RNAi caused endogenous Axin elevation (Fig 4.6B), increased numbers of Arm+/Pros− stem cells (Fig 4.6C), increased number of pH3+ cells (Fig 4.6D) and increased upd3>GFP reporter activity (Fig 4.6E). These results are consistent with in vitro function of ubcD1 in promoting TNKS and Axin degradation.

4.3.4 TNKS-mediated ADP-ribosylation can provide a molecular platform for rapid degradation of PARsylated proteins

My hypothesis suggests that ADP-ribosylation might be a molecular scaffold to concentrate the 26S proteasome locally and accelerate the degradation of TNKS or its PARsylated substrates. Therefore, I tested if TNKS co-localized with the 26S proteasome in the cell. To accomplish this, I miss-expressed UAS-Flag-Tnks in adult fly midguts under Myo1A driver, and dissected midguts were incubated with a fluorescent probe (Me4BodipyFL-
AhxLeu3VS) for 26S proteasome labeling (Berkers et al., 2007). I found that Flag-tagged TNKS co-localized with the 26S proteasome in ECs (Fig 4.7A). To address the importance of the PARsylation scaffold for the proteasome-TNKS interaction, I treated TNKS-expressing midguts with a small molecule inhibitor of TNKS, XAV939. I observed that both Flag-TNKS and 26S proteasomes changed their localizations upon XAV939 treatment (Fig 4.7B). TNKS protein level increased whereas the cytoplasmic proteasome localization diminished. As a conclusion, this result suggested that PARsylation might be a signal for proteasome recruitment into a local environment.

One possible mechanism by which the proteasome might be transported to the ADP-ribosylation scaffold is through an adaptor protein. Our lab previously demonstrated that PI31 physiologically required for the optimal 26S proteasome activity in vivo. Loss-of PI31 causes a reduction of protein breakdown in several tissues and organismal lethality (Bader et al., 2011). PI31 is regulated by several different mechanisms including TNKS-dependent ADP-ribosylation and p38-mediated phosphorylation. ADP-ribosylation of PI31, for instance, alters its binding affinity for 20S proteasome alpha subunits and 19S assembly chaperones. As a consequence, the 26S proteasome assembly and activity are enhanced (Park and Steller, 2013). Furthermore, our lab has recently demonstrated that PI31 plays a role as an adaptor protein in proteasome transport, required for synaptic structure and function (Liu et al., 2019). PI31 binds directly to proteasomes with dynein light chain proteins (DYNLL1/2). PI31 depletion, therefore, inhibits proteasome movements in axons. Finally, p38 MAP kinase-mediated phosphorylation of PI31 promotes its binding to DYNLL1/2. Phosphorylation deficient PI31 reduces proteasome transport in axons (Liu et al., 2019). I, therefore, tested if PI31 might have a role in ADP-ribosylation-dependent protein destruction.
Figure 4.7 The co-localization of TNKS and proteasomes depends on PARP activity TNKS.
A- ECs cells expressed Flag-Tnks under Myo1A driver. Dissected midguts were incubated with a fluorescent probe (Me4BodipyFL-AhxLeu3VS) for 26S proteasome labeling in a conditioned media for 3h. B- Dissected midguts were treated with 20µM XAV939 for 3h. 7-day-old females were studied. After 4% PFA fixation, midguts were stained against Flag and analyzed by confocal microscope. Scale bar: 10µm.
Figure 4.7

A

Flag-Tankyrase  Active 26S Proteasome  DAPI  Merged

B

Flag-Tankyrase  Active 26S proteasome  DAPI  Merged

DMSO

XAV939
I showed TNKS ADP-ribosylated PI31 in vitro, and this modification was sensitive to XAV939 treatment and hNUDT16 cleavage (Fig 4.8A). While TNKS-mediated ADP-ribosylation is mainly related to the protein breakdown through the 26S proteasome, loss-of Tnks did not result in PI31 elevation (Fig 4.8B), suggesting that ADP-ribosylation is not a degradation signal for PI31. I also demonstrated the whole protein lysate of PI31 mutant larvae did not display Axin accumulation (Fig 4.8C). To examine the function of PI31 in Armadillo degradation in vivo, I generated mosaic animals with PI31⁻/⁻ cells by using Flipase/FRT system. PI31 inactivation in mutant clones did not affect Armadillo destruction in wing imaginal discs (Fig 4.8D), thereby suggesting that PI31 might not affect in PARsylation-mediated protein turn-over in all tissues.

Another possible mechanism to explain how the 26S proteasome might affect rapid degradation of PARsylated proteins was that the 26S proteasome might bind to a PAR scaffold through PAR-binding module in its subunits. I found that the 19S subunits Rpt1, 2, 3, and 6 were significantly co-immunoprecipitated with Flag-tagged TNKS in S2R+ cells (Table 4.1). When their amino acid sequences were aligned with the canonical PBM, the Rpt proteins of 19S regulatory particle had many essential core lysine-arginine amino acids (RR, KK or RK) but not the β- or α-subunits of the 20S proteasome did not. Detailed analyses revealed that Rpt2, 3 and 6 had several canonical PBMs (Fig 4.9). I, therefore, suggest that PBMs on Rpt subunits may facilitate the binding of 26S proteasomes to PARsylation scaffold. As a consequence, PARsylation can bring TNKS, Iduna, ubcD1, substrates, and the 26S proteasome together into close proximity, thereby accelerating the breakdown of substrates (Fig 4.10).
Figure 4.8 PI31 does not affect in Axin and Armadillo degradation in L3 larvae. A- TNKS ADP-ribosylated PI31 in vitro. His-tagged *Drosophila* PI31 was expressed and purified from *E. coli*. ADP-ribosylation of PI31 was a covalent modification that was sensitive to XAV939 treatment and hNUDT16 digestion. B- PI31 ADP-ribosylation is not a proteolysis signal in *Drosophila*. Tnks inactivation did not result in PI31 elevation. 7-day-old males were lysed for PI31 immunoblotting (IB: α-dPI31). C- Depletion of *PI31* does not affect endogenous Axin levels in L3 larvae. *PI31*<sup>+/−</sup>; *BAC-Axin-V5* or +/+; *BAC-Axin-V5* larvae were lysed for immunoblotting (IB: α-V5). α-Tubulin and β-actin were loading controls. D- *FRT42D, PI31* mutant clones were analyzed for Armadillo levels. Dissected 3<sup>rd</sup> instar wing imaginal discs were stained with an Armadillo antibody. I did not observe an elevation of Armadillo in *PI31*<sup>+/−</sup> mutant clones. Scale bar: 25µm.
Figure 4.8

A

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PADPR TNKS, WB: α-PAR

ADPR dPI31
Flag TNKS
His dPI31
His hNUDT16

B

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C

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D

RFP<sup>NLS</sup>, Armadillo, DAPI, Merged

L3 larvae wing imaginal disc
Figure 4.9 Rpt2, 3 and 6 subunits of the 19S regulatory particles of the proteasome have several canonical PAR-binding motifs. The canonical PBM consists of core lysine and arginine residues: AVILMFYM$_{1}$-X$_{2}$-KR$_{3}$-X$_{4}$-AVILMFYW$_{5}$-AVILMFYW$_{6}$-KR$_{7}$-KR$_{8}$-AVILMFYW$_{9}$-AVILMFYW$_{10}$. Core residues were highlighted with pink. The canonical PBM-containing peptides of Rpt2, 3 and 6 were colored as red.
Figure 4.10 ADP-ribosylation by TNKS provides a molecular scaffold that brings substrates, E2 and E3 ubiquitin ligases and proteasomes into close proximity, thereby accelerating target protein degradation. I propose that TNKS forms a complex with Iduna, which is inactive when bound to non-PARylated TNKS in the cell. After substrate binding to TNKS and subsequent PARylation, Iduna binds to PAR. This binding causes a conformational change in the RING domain of Iduna, which activates its ubiquitin ligase activity, enabling the poly-ubiquitinylation of the substrate. The ubcD1 ubiquitin-conjugating enzyme promotes ubiquitin ligase function of Iduna by binding to ADP-ribose. As a consequence, TNKS may increase its PARsylation activity to assemble PAR scaffold. Finally, 26S proteasomes may be recruited to the scaffold and concentrated in a local environment for rapid destruction of the target protein.
4.4 Material and Methods

Fly stocks: Flies were kept at a 12-hour light/dark cycle. All crosses were performed at 22-25°C unless stated otherwise. The following fly stocks were used for this study (Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VDRC) number (#) given in parentheses):

The stocks used in here: esg-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), BAC-AxinV5 (a gift of Jean-Paul Vincent, Gerlach et al., 2014), FRT 82B, Axn\(^h\), BAC-AxinV5 (a gift of Jean-Paul Vincent, Gerlach et al., 2014), UAS-GFP-Axin (BDSC# 7224), Myo1A-Gal4, tub-Gal80ts, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), upd3-Gal4, UAS-GFP (a gift of Dr. Norbert Perrimon; Micchelli and Perrimon, 2006), Tnks\(^{503}\) (a gift of Dr. Yahsi Ahmed, Wang et al., 2016), Tnks\(^{19}\) (a gift of Dr. Yahsi Ahmed, Wang et al., 2016), PI31\(^{-/-}\) (Bader et al., 2009), UAS-ubcD1-HA (Ryoo et al., 2002), UAS-Flag-Tnks (Gultekin and Steller, 2019), UAS-Tnks-HA (a gift of Dr. Yahsi Ahmed, Wang et al., 2016), UAS-CG8786/dIduna RNAi#1 (BDSC# 40882), UAS-CG8786/dIduna RNAi#2 (VDRC#43533), UAS-CG8786/dIduna RNAi#3 (VDRC#36028), UAS-CG8786/dIduna RNAi#4 (VDRC#36029), and white RNAi (BDSC#33623), ubcD1 RNAi (BDSC#31875), ubcD1 RNAi (BDSC#35431), morgue RNAi (BDSC#31059). The rest of Drosophila lines, which were studied here, were obtained from Steller Lab stocks. Oregon R flies were used as control and only adult female flies were analyzed in this study.

Clone analyses and RNAi experiments: Please refer to Chapter 2, Material and methods.

Cell culture: Please refer to Chapter 2, Material and methods.
Western blot analyses: Please refer to Chapter 2, Material and methods.

Primary antibodies: anti-dPI31 (rabbit, generated in this study, 1/2500), anti-V5 (1/1000, Abcam, ab27671), anti-Flag-HRP (1/1000, Sigma-Aldrich, A8592), anti-Flag (1/1000, CST, D6W5B 14793), anti-GST HRP (1/1000, GenScript, A00866-100), anti-PAR (1/1000, Trevigen, 4335-MC-100), anti-Myc tag (1/1000, CST, 9B11, 2276), anti-Axin Drosophila (Santa Cruz, dT20, sc15685), anti-tubulin DM1A clone (1/1000, Sigma-Aldrich, T9026), mouse anti-GFP-HRP (1/2500, clone B2, Santa Cruz Biotechnology, sc-9961-HRP), rabbit anti-β-Actin-HRP (1/5000, mouse anti-Flag (Sigma, M2-F1804, 1:5000), rabbit anti-V5 (Abcam, ab27671, 1:1000), mouse anti-Arm (Wang et al., 2016; N2 7A1, DSHB, 1:50), mouse anti-Prospero (Wang et al., 2016; MR1A, DSHB, 1/50), mouse anti-GFP (GFP-12A6, DSHB, 1/100), mouse anti-β-galactosidase (Tian et al., 2016); 401A, DSHB, 1/100), mouse anti-Delta (Wang et al., 2016; C594.9B, DSHB, 1/100), rabbit anti-pha8-Histone3 (Wang et al., 2016; 06-570, Millipore, 1/1000).

The secondary antibodies were goat anti-mouse-Alexa 488 plus (Thermo Fisher Scientific, A32723), goat anti-mouse-Alexa 568 (Thermo Fisher Scientific, A11031), goat rabbit-Alexa 546 (Thermo Fisher Scientific, A11035), goat rabbit-Alexa 488 (Thermo Fisher Scientific, A11034), goat anti-rabbit-Alexa 633 (Thermo Fisher Scientific, A21071) and used at 1/1000.
**Immunoprecipitation:** Please refer to Chapter 2, Material and methods.

**Recombinant protein purification from S2R+ cells:** Please refer to Chapter 2, Material and methods.

**Antibody production against Drosophila PI31:** To generate a polyclonal antibody against Drosophila PI31, GST-PI31 was expressed in BL21 Star (DE3) *E. coli* cells (Thermo Fisher Scientific, C601003), and purified using Glutathione Sepharose 4B beads (GE Healthcare, 17-0756-01). The purified protein was injected into rabbits and the antisera were collected (Cocalico).

**In vitro ADP-ribosylation:** 20 micrograms of Flag-tagged *Drosophila* TNKS protein (purified from S2R+ cells) were automodified in 100 µL of a reaction buffer containing 100mM Tris-HCl pH 8.0, 10mM MgCl2, 10mM DTT, 25µM NAD+ for 1h at 24-25°C.

**In vitro ubiquitinylation assays:** For Iduna auto-ubiquitinylation, 50ng E1, 100ng T7–ubcD1, 700ng His-Iduna, and PAR were used in each reaction. For TNKS ubiquitinylation, 750ng Flag-tagged TNKS (purified from S2R+ cells, *in vitro* ADP-ribosylated and not) 50ng E1, 100ng T7–ubcD1, 100ng His-Iduna, and PAR were used in each reaction. *In vitro* ubiquitinylation assays were carried out in a total volume of 20 µl. The reaction mixture contained also 40mM Tris-HCl at pH 7.6, 5mM MgCl2, 1mM dithiothreitol, 5mg GST-ubiquitin, 100ng ubiquitin–aldehyde, 2mM ATP. Reactions were incubated for 1h at 37 °C before addition of sample buffer and resolution on 4-20 % SDS–PAGE gels. Finally, Western blotting was carried out.

**Quantification and statistics:** Please refer to Chapter 2, Material and methods.
5 DISCUSSION

5.1 Iduna regulates stem cell proliferation and intestinal homeostasis

We investigated the *in vivo* function of *Iduna* and identified a critical role of this enzyme for the control of stem cell proliferation in the *Drosophila* midgut. Mammalian Iduna is an unusual E3 ubiquitin ligase that specifically binds to and poly-ubiquitylates ADP-ribosylated substrates to promote their rapid degradation by the proteasome. However, the physiological function of Iduna remains largely unclear.

Here, I generated *Drosophila Iduna*-null-mutants and used them to show that Iduna has a crucial *in vivo* function for the degradation of ADP-ribosylated TNKS and Axin to control stem cell proliferation. In particular, I focused on the role of *Iduna* in the *Drosophila* midgut. I found that *Iduna* inactivation caused a slight but significant increase in Axin protein levels in ECs, which in turn caused over-proliferation of ISCs. This non-cell autonomous effect on stem cell proliferation is depended on UPD2-UPD3 cytokines that are secreted from ECs. These findings suggest a model in which loss of *Iduna* function, which decreases the Wingless pathway activity due to elevated Axin levels in ECs, in turn causes increased secretion of UPD2-3 from these cells to activate the JAK-STAT pathway in ISCs. Importantly, a 50% reduction in *Axin* gene dosage blocked the over-proliferation of stem cells in *Iduna* mutants, demonstrating the necessity to tightly regulated Axin levels in this system. Whereas many other cell types appear to tolerate fluctuations in the amount of Axin protein, proper Wingless signaling in the *Drosophila* midgut appears to critically depend on restricting Axin levels by Iduna.

The activity of Iduna depends on binding to ADP-ribosylated substrates via its WWE domain. Recognition and binding to its ADP-ribosylated target proteins change the
structural confirmation of Iduna. Subsequently, Iduna is activated to ubiquitylate its targets for proteasome-mediated degradation. It was previously reported that TNKS forms a tight complex with Iduna to control the proteolysis of target proteins (DaRosa et al., 2014). I could not detect any obvious morphological differences between Iduna mutants and wild type. Although this may seem somewhat surprising, it is consistent with complete inactivation of Tnks in Drosophila, which also causes no overt abnormalities (Feng et al., 2014; Wang et al., 2016 a,b; Yang et al., 2016). As for Iduna, Tnks mutants have no obvious effects on wing development and the expression of Wingless target genes in larval wing discs, despite the fact that Axin levels are increased (Feng et al., 2014; Wang et al., 2016a and 2016b; Yang et al., 2016). Our interpretation of these findings is that most tissues can tolerate relatively modest (2-3-fold) changes of Axin. For example, it appears that a greater than 3-fold increase of endogenous Axin is required for functional consequences of altered Wingless signaling in Drosophila embryos (Yang et al., 2016) and 3-9-fold changes are needed in wing discs (Wang et al., 2016a). On the other hand, the Drosophila midgut appears much more sensitive to reduced Wingless signaling.

An independent study demonstrated that inactivation of Drosophila Tnks led to increased Axin protein accumulation in the Drosophila midgut and promoted ISC proliferation (Wang et al., 2016). These results are consistent with previously reported cell-based studies suggesting that Iduna mediates TNKS-dependent degradation of Axin and thereby positively regulates Wnt signaling (Huang et al., 2009; Croy et al., 2016; Callow et al., 2011). On the other hand, it is notable that inactivation of two highly diverse types of enzymes, Tnks, a PARP, and Iduna, an E3 ubiquitin ligase, produced
similar phenotypes. Both TNKS and Iduna have many other targets outside the Wnt-pathway, and based on biochemical observations, it has been proposed that they may play roles in DNA repair, telomere length, vesicle trafficking, Notch-signaling, centrosome maturation, neuronal protection and cell death (Bai, 2012; Gibson and Kraus, 2012; Riffell et al., 2012). However, Iduna and Tnks mutant flies are viable and do not show any obvious defects under normal growth conditions. This indicates that the major non-redundant physiological function of both Tnks and Iduna in Drosophila is to regulate Wingless-mediated intestinal stem cell proliferation, and provides physiological evidence for the idea that the function of both proteins is indeed tightly coupled. In addition, our study identifies a role of UPD-Domeless in this pathway. These results may have implications for the regulation of this highly conserved pathway in mammals. For example, conditional inactivation of Iduna in mouse bones leads to increased numbers of osteoclasts and inflammation (Matsumoto et al., 2017a). In this system, down-regulation of Iduna leads to accumulation of Axin1 and 3BP2. This, in turn, attenuates β-catenin degradation and activates SRC kinase, respectively, thereby promoting the release of inflammatory cytokines in the bone (Matsumoto et al., 2017a). On the other hand, Iduna depletion reduces proliferation of osteoblasts and promotes adipogenesis in the mouse skeleton (Matsumoto et al., 2017b). Despite the obvious differences between mammalian bone and the Drosophila midgut, both systems show overall striking similarities in the use of TNKS/Iduna to restrict Axin levels to achieve proper levels of Wnt/β-catenin signaling during tissue homeostasis. Finally, my study indicates that Axin may have a more general function as a scaffold protein that recruits multiple proteins and permits a crosstalk with other pathways that modulate Wnt/β-catenin signaling.
5.2 Iduna can play a role in energy metabolism in Drosophila

The PARsylation activity of TNKS regulates diverse physiological processes including energy metabolism. PARP activity consumes NAD+ as a co-substrate to post-translationally modify various acceptor proteins including TNKS itself. PARsylation by TNKS often tags the acceptors for ubiquitylation and proteasomal degradation. In insulin-secreting cells, glucose and fatty acid consumptions stimulate the auto-PARsylation of TNKS; consequently, its breakdown is promoted. Elevation of NAD+ levels primarily mediates this glucose effect on TNKS (Zhong et al., 2015). Furthermore, TNKS binds to IRAP1 (insulin-responsive aminopeptidase) and co-localizes with GLUT-4 vesicles near the Golgi in 3T3-L1 adipocytes (Chi and Lodish, 2000). Because of its Golgi localization, TNKS can have an impact on the exocytosis of specific cargos in adipocytes. Upon insulin stimulation, MAP kinase phosphorylates TNKS, which in turn enhances its PARP activity in adipocytes. As a result, it is suggested that TNKS-mediated PARsylation of Golgi-associated proteins may affect the sorting or stability of GLUT4 vesicles (Chi and Lodish, 2000). Moreover, genetic depletion of Tnks in adipose tissue increases glucose tolerance and adiponectin levels in female mice (Zhong et al., 2016). Global TNKS knockouts also share these phenotypes (Yeh et al., 2009). The similarity between global knockout and tissue-specific deletion suggest that the impact of TNKS on systemic metabolism largely comes from its PARsylation activity in adipocytes (Zhong et al., 2016).

Mice with loss of Iduna within the osteoblast lineage have increased fat stores and are glucose intolerant with severe osteopenia because of defective osteoblastogenesis and
subsequent impaired osteocalcin production. Hence, Iduna controls bone production, glucose, and lipid metabolism (Matsumoto et al., 2017b).

TNKS also influences lifespan, stress tolerance and energy storage in Drosophila (Li et al., 2018). The knockdown of Tnks in muscle causes similar effects to those of Tnks-null mutants and its ubiquitous knockdown. They exhibit shortened lifespan, impaired climbing ability, increased sensitivity to oxidative stress and starvation, and reduced TAG and glycogen storage (Li et al., 2018). Similar to TNKS, Iduna affects longevity in Drosophila. Iduna mutants have slightly shorter lifespans than their wild type controls. They display increased mortality upon reduced nutrient diet. Remarkably, Iduna depletion reduces stored lipids in abdominal fat body cells upon starvation. I have shown that Iduna-null flies steadily lost their fat stores during a 24h starvation, while wild type control flies were able to sustain their fat reservoirs for 24h. These results point out another role of Iduna in promoting survival under stress conditions. I, therefore, suggest that Iduna may have an essential function(s) in energy metabolism as well. The exact mechanism by which starvation caused increased mortality in Iduna mutants remains to be determined.

5.3 Lysine 598 in TNKS affects longevity in Drosophila

I explored the importance of PARsylation-mediated proteolysis by focusing on TNKS-mediated ADP-ribosylation and the E3 ubiquitin ligase function of Iduna. PARsylation is a degradation signal for TNKS and Axin. However, the ribosylation and the PARsylation-dependent ubiquitylation sites that target TNKS and Axin for degradation are not yet known. With an MS-based approach, I reveal that K598 residue is
one of the ADP-ribose acceptor sides in *Drosophila* TNKS. *In vitro* ADP-ribosylation assays suggested that K598A might be a loss-of-function mutation of *Tnks* since it was unable to ADP-ribosylate *Drosophila* Axin as well as itself *in vitro*. However, I found that K598A mutant was auto-ADP-ribosylated and degraded in S2R+ cells. Lysine 598 is located in the ARC4 domain, which is conserved from flies to human. This residue corresponds to lysine 604 in human TNKS2. It was previously demonstrated that K604 in human TNKS2 might be essential for Axin1-binding as well as its degradation. Therefore, it was suggested K604TNKS might form strong salt bridge interactions between 7th and 8th glutamine residues in Axin’s TBM. Although there are no crystal structures for ARC4 to bind Axin1/2 yet, recent reports have demonstrated that ARC1, 3 and 5 repeats in human TNKS2 are necessary for Axin recognition and binding.

TNKS is a negative regulator of Axin. As a result, *Tnks*-null flies have elevated Axin in *Drosophila*. Loss-of *Tnks* promotes stem cell proliferation through non-cell autonomous JAK-STAT activation. As a consequence, there are more stem and progenitor cells in *Drosophila* midguts. On the other hand, *Tnks*K598A knock-in flies do not promote the cell division of ISCs. Hence, *Tnks*K598A is not a complete loss-of-function mutation of *Tnks*. Taken together, I suggest that K598-ADP ribosylation can have an essential function(s) for other protein(s) rather than Axin.

*Tnks*K598A adult flies are viable, fertile, and have no apparent morphological defects. However, *Tnks*K598A flies live significantly shorter than their control flies. *Tnks*K598A adult flies also decline their climbing abilities with age. Similarly, *Tnks*-null flies have prominently shorter lifespan. Loss-of *Tnks* or its muscle-specific inactivation impairs longevity, stress tolerance, and energy storage in adult flies through regulation of
the JNK pathway. TNKS PARsylates JNK, which positively controls JNK signaling. PARsylation is not a degradation signal for JNK. Instead, TNKS triggers degradation-independent K63-ubiquitination of JNK to promote its kinase activity in *Drosophila* (Li et al., 2019). Taken together, I suggest that K598 in TNKS may have a role in JNK signaling in the *Drosophila* muscle.

I finally conducted a Co-IP-MS to identify the most affected binding protein(s) of TNKS when lysine 598 was mutated to alanine. I, therefore, investigated that 11 proteins that prominently lost their binding-affinities to TNKS due to this mutation. As a result, I suggest that K598 may have various physiological functions together with these binding partners. For instance, RH07106p is the most affected binding-partner of TNKS. RH07106p is an Hsp70-binding protein. It has a putative transmembrane domain, and is expected to localize on the endoplasmic reticulum membrane. Its molecular functions are not known yet. Likewise, K598A TNKS does not pull down Snapin, a component of the biogenesis of lysosome-related organelles complex-1 (BLOC-1). It functions in different biological processes including intracellular protein transport, synaptic vehicles priming, and neurotransmitter release.

In conclusion, my results point out that lysine 598 can be essential to interact with several binding-partners of TNKS. Further molecular studies on the affected interaction partners of TNKS can be helpful to further investigate the physiological function(s) of lysine 598.
5.4 ubcD1 ubiquitin-conjugating enzyme promotes ubiquitin ligase activity of Iduna

I described the ubiquitin-conjugating function of ubcD1 for Iduna-dependent ubiquitylation. Loss-of ubcD1 results in elevations of Iduna, TNKS and Axin proteins in *Drosophila*. ubcD1 boosts auto-ubiquitylation of Iduna in the presence of PAR and promotes degradation of Iduna, TNKS and Axin *in vivo*. ubcD1 can bind to PAR scaffold through its PBMs.

I also showed that Flag-tagged TNKS significantly co-immunoprecipitates with Ben, morgue, Usp10, and Usp30 in S2R+ cells. Similar to ubcD1, Ben and Morgue are ubiquitin-conjugating enzymes. It is possible that they may have functions on TNKS-dependent protein breakdown although morgue-RNAi does not have an impact on the stability TNKS itself. On the other hand, Usp10 and Usp30 are ubiquitin-specific proteases, which can remove ubiquitin chains and stabilize proteins. USP25 positively regulates Wnt signaling by mediating the deubiquitylation of TNKS in human cells. Conversely, si-RNA-dependent USP25 deficiency promotes TNKS degradation, which in turn causes suppression of Wnt signaling (Xu et al., 2017). However, the functions of usp10 and usp30 on TNKS or its substrates are not known yet. Future work, therefore, is required to address the contribution of these E2 ubiquitin ligases and deubiquitinating enzymes for TNKS-mediated proteolysis.
5.5 ADP-ribosylation can provide a molecular scaffold for TNKS-mediated rapid protein degradation

Finally, I provide evidence that PARsylation can recruit necessary proteins including the 26S proteasome in a local environment to maintain rapid protein degradation. I found that TNKS can colocalize with the 26S proteasome. Reduction of PARsylation by XAV939 blocks cytoplasmic co-localization between TNKS and the 26S proteasome. This result suggests that the 26S proteasome by itself may bind to PAR polymers. Unlike the 20S core particles, the 19S regulatory Rpt proteins consist of many lysine-arginine enrich peptides but Rpt2, 3 and 6 have canonical PBM.

PAR-binding proteins recognize PAR polymers, thereby, driving the localization of proteins and assembly of protein complexes. PARP1-auto-PARsylation provides a molecular platform to recruit all essential proteins into close proximity in response to DNA damage, rRNA biogenesis, and heat shock. Similarly, TNKS-mediated PARsylation is essential for stress granule assembly. TNKS-mediated PARsylation promotes TDP45 liquid-liquid phase separation and protect TDP45 from ALS-associated phosphorylation (Duan et al., 2018; McGurk et al., 2018).

Since Nature uses PAR scaffold in different concepts, I propose a mechanism, by which PARsylation can bring together TNKS, target proteins, E2 and E3 ubiquitin ligases such as ubcD1 and Iduna, respectively and the 26S proteasome into close proximity for accelerating the breakdown of PARsylated proteins. Based on my model, TNKS forms a complex with Iduna, which is inactive when bound to non-PARylated TNKS in the cell. After substrate recognition and binding to the complex, TNKS starts to ADP-ribosylate the substrate. When the target protein has multi-ADP-ribose moieties, Iduna binds an
internal unit of PAR. This binding causes a conformational change in the RING domain of Iduna, which activates its ubiquitin ligase activity, enabling the poly-ubiquitylation of the substrate. As a consequence, TNKS is promoted for its PARsylation activity to assemble PAR scaffold. Finally, 26S proteasomes can be recruited to the scaffold. As a result, rapid protein degradation takes place.

My data has led to a two-step model for regulation of protein degradation by ADP-ribosylation whereby TNKS regulates the formation of the cytoplasmic scaffold of ADP-ribosylation and poly-ADP-ribose regulates the recruitment of 26 proteasomes to the local environment for degradation of target proteins. I can suggest the scaffold model can be a very dynamic and follow the rules of liquid-liquid phase separation. Under regular conditions, TNKS with Iduna can form liquid condensates that can mature, solidify, and undergo liquid-to-solid transitions—or can nucleate aggregation. The oligomerization of TNKS through SAM domain promotes its poly-ADP-ribose polymerase activity. The negatively charged poly-ADP-ribose scaffold, therefore, can promote liquid-liquid phase separation for TNKS-Iduna degradation complex. Importantly, a small molecule inhibitor of TNKS, XAV939 reduces the co-localization of poly-ADP-ribose and proteasomes in the cytoplasm. Thus, poly-ADP-ribosylation may modulate liquid-liquid phase separation, which can accelerate the target protein degradation. Future studies will determine whether TNKS-Iduna complex, ribosylated target proteins, and 26S proteasome can co-phase separate in vitro.
5.6 Future perspectives of this thesis

5.6.1 Insights into the \textit{in vivo} functions of Iduna in \textit{Drosophila}

Several \textit{in vivo} functions of Iduna in \textit{Drosophila} were uncovered in this study including its physiological impacts on midgut stem cells and intestinal homeostasis. However, results from this study imply that Iduna has potential regulatory roles in lipid metabolism. Yet, the cause of increased mortality of Iduna mutants upon 5% sucrose diet remains to be uncovered. Iduna mutants also reduce stored lipids in abdominal fat body cells upon 12h or 24h starvation. Iduna-null flies are not able to restore their lipids into fat bodies during a 24h starvation, while wild type flies can. These results demonstrate that Iduna has an important role in promoting survival during starvation. To uncover the physiological relevance of Iduna in \textit{Drosophila}, I performed an RNA-sequencing experiment from the dissected intestines of 15-day-old Iduna mutant and control flies. I found that a pancreatic-like lipase, \textit{CG6271} was significantly down-regulated in normal circumstances (Appendix). Both FlyAtlas and FlyGut-seq databases suggest that the expression of \textit{CG6271} gene is high in enteroendocrine cells in the \textit{Drosophila} midgut (Chintapalli et al., 2007; Buchon et al., 2013; Marianes et al., 2013; Dutta et al., 2013). \textit{CG6271} depletion by RNAi under Prospero-Gal4 driver leads to lethality in fly larvae (Data not showed). However, the physiological functions of CG6271 remain to be determined. I suggest that CG6271 may play a role in lipid metabolism and further molecular and cellular studies will address the function of Iduna in lipid metabolism.
5.6.2 Age-related physiological roles of lysine 598 in *Drosophila* TNKS

This study has led to the identification of one of the first ADP-ribosylation acceptor residues in *Drosophila* TNKS. I found that *Tnks*<sup>K598A</sup> adult flies are viable, and fertile without any obvious phenotypic defects. However, *Tnks*<sup>K598A</sup> mutants live significantly shorter than their controls. *Tnks*<sup>K598A</sup> adults also decline their climbing abilities, JNK activation and global protein poly-ADP-ribosylation with age. Finally, I showed that FLAG-tagged K598A TNKS prominently lose its binding-affinity to 11 proteins including RH07106p Hsp70 binding protein, Caper, Snapin, Receptor-mediated endocytosis protein 6 homolog, Prx5, RnpS1, IP12463p putative cADPR synthase, Exu, A1Z6G6, U2af50, Pst.

One of the surprising binding-partner of TNKS is Snapin that does not interact with K598A TNKS. Snapin participates in the biogenesis of lysosome-related organelles. It is a component of the SNARE complex of proteins that is required for synaptic vesicle docking and fusion. Snapin is an essential protein for presynaptic plasticity. Hence, Snapin has important functions including intracellular protein transport, synaptic vehicles priming, and neurotransmitter release (Starcevuc et al., 2004; Ilardi et al., 1999).

Since TNKS is a positive regulator in the JNK signaling pathway, changes in the activity of JNK signaling can suppress or enhance phenotypes of *Tnks* mutant flies. Therefore, we need further studies to investigate how *Tnks*<sup>K598A</sup> mutation affects JNK signaling during aging and different stress responses including starvation, bacterial infections, and chemical toxins. Furthermore, these phenotypes in *Tnks*<sup>K598A</sup> mutants suggest that there might be problems in organismal metabolism during aging. Various metabolic tissues including muscle, fat body, neuron, and intestine can sense nutrients
and environmental inputs to affect aging-associated cellular functions. Ectopic expression of UAS-wild type Tnks in these tissues under tissue-specific drivers can determine the major tissue(s) in which $Tnks^{K598A}$ play its physiological roles. It was previously shown that $Tnks$ knock-down in the muscle with Mef2-Gal4 causes shortened lifespan, impaired climbing ability similar to $Tnks^{K598A}$ mutant flies. Finally, comparative interactome between wild type and K598A result suggests Snapin may be a molecular target. Hence, the muscle may be important to further understand the physiological role(s) of K598 residue in Drosophila. Cellular and molecular investigations need to be performed on neuromuscular junctions and muscle of $Tnks^{K598A}$ mutants. Complementary electrophysiology experiments need to be can be helpful to further investigate the physiological function(s) of lysine 598 in the muscle.
Figure 6.1 RNA sequencing indicates that *Iduna* depletion down-regulates *CG6271*. 15-day-old adult female flies were dissected for their midguts and directly homogenized in 1 ml TRIzol (Life Technologies). Total RNA was isolated according to the manufacturer’s protocol. The Rockefeller University Genomics Core Facility performed RNA quality control test before RNA-sequencing. RNA-seq libraries were generated at The Rockefeller University Genomics Resource Center. Files were delivered in fastq format. For data analysis, reads were aligned against the *D. melanogaster* genome dm6 with BWA or tophat2 (for poly(A) and total RNA-seq) in standard settings. **A-** Volcano plot of significantly and differentially expressed genes sanity-check the DGE is to see how all genes behave across conditions using a standard "volcano plot". This plot is a special case of the scatter plot where each gene is represented by an individual dot, X-axis shows the degree of fold-change in logarithmic base and the Y-axis is the minus log10 transformation of the raw p-value of statistical significance. The horizontal line shows the significance cut-off hence all genes in the top-left corner are significantly down-regulated and the top-right ones are significantly up-regulated ones. **B-** A list of Significantly and differentially expressed genes (DGE) (FDR 10%). The logFC column is the well-known "log fold-change" value. Positive values represent up-regulation and negative ones down-regulation. The columns AveExpr, t, and B are mostly regarding the DGE statistics and are not informative in terms of the biology. The table is sorted based on the raw p-value and can be considered as an ordered list of genes where the top one is the most relevant. **C-** A heat map of significantly and differentially expressed genes. We showed the number of reads mapped to each gene
using a log-transform for a better color scheme and we will do this for all significant genes. This is a bit hard to interpret by eye, but we definitely would like to see the same conditions clustering together and as a bonus, we might get some interesting groupings of the genes. **D-** *CG6271* is highly expressed in the *Drosophila* midgut (FlyAtlas database). **E-** *CG6271* is expression is enriched in enteroendocrine cells in the R3 region of the midgut. It has a high expression in the visceral muscle in the R2 region of the midgut (FlyGut-Seq Database). **E-** CG6271 is a pancreatic-like lipase in *Drosophila*, which shares 47% similarity with human LPL protein.
Figure 6.1

A  Mutant vs Control

B

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Figure 6.1
### Figure 6.1

#### D

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<tr>
<th>Tissue</th>
<th>mRNA Signal</th>
<th>Present Call</th>
<th>Enrichment</th>
<th>Affy Call</th>
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<td>0 ± 0</td>
<td>0 of 4</td>
<td>0.00</td>
<td>None</td>
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<tr>
<td>Head</td>
<td>2 ± 1</td>
<td>0 of 4</td>
<td>0.00</td>
<td>None</td>
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<tr>
<td>Eye</td>
<td>3 ± 1</td>
<td>0 of 4</td>
<td>0.03</td>
<td>None</td>
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<tr>
<td>Thoracicoabdominal ganglion</td>
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<td>0.00</td>
<td>None</td>
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<tr>
<td>Salivary gland</td>
<td>1 ± 0</td>
<td>0 of 4</td>
<td>0.02</td>
<td>None</td>
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<tr>
<td>Crop</td>
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<td>0.00</td>
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<tr>
<td>Midgut</td>
<td>2923 ± 168</td>
<td>4 of 4</td>
<td>28.20</td>
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<td>Tubule</td>
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<td>0.00</td>
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<td>Fat body</td>
<td>2 ± 2</td>
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<td>0.03</td>
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<td>Testis</td>
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<td>0.00</td>
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<tr>
<td>Male accessory glands</td>
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<td>0.00</td>
<td>None</td>
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<tr>
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<td>0.01</td>
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<tr>
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</tr>
<tr>
<td>Larval CNS</td>
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<td>0 of 4</td>
<td>0.01</td>
<td>None</td>
</tr>
<tr>
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<td>0 of 4</td>
<td>0.02</td>
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<tr>
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<td>0.00</td>
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<tr>
<td>Larval hindgut</td>
<td>1 ± 0</td>
<td>0 of 4</td>
<td>0.01</td>
<td>None</td>
</tr>
<tr>
<td>Larval fat body</td>
<td>2 ± 0</td>
<td>0 of 4</td>
<td>0.00</td>
<td>None</td>
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<tr>
<td>Larval trachea</td>
<td>0 ± 0</td>
<td>0 of 4</td>
<td>0.00</td>
<td>None</td>
</tr>
<tr>
<td>Larval carcass</td>
<td>0 ± 0</td>
<td>0 of 4</td>
<td>0.01</td>
<td>None</td>
</tr>
<tr>
<td>S2 cells (growing)</td>
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<td>0.01</td>
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<tr>
<td>Whole fly</td>
<td>103 ± 47</td>
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#### E

**Combined expression -**

![Combined expression graph](link)
Figure 6.1

Protein Alignment: CG6271 and LPL

<table>
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<tr>
<th>Sequence 1: NP_651526.1</th>
<th>Gene: CG6271</th>
<th>FlyBASEID: FBgn0039476</th>
<th>Length: 341</th>
<th>Species: Drosophila melanogaster</th>
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<td>WLEAQRKDC-</td>
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<td>14</td>
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<td>79</td>
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<td>Fly</td>
<td>164</td>
<td>PLEDNIEKKEVTVIIRASIQFTDQSONTVLPLTVSRFKEFL-</td>
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Gene | Sequence | Domain               | Region | External ID | Identity |
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<tbody>
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<td>CG6271</td>
<td>NP_651526.1</td>
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<td>57-337</td>
<td>CDD:278576</td>
<td>94/322 (29%)</td>
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<td>Lipase</td>
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<td>Heparin-binding, (ECO:0000250)</td>
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</tbody>
</table>

Blue background indicates that the domain is not in the aligned region.
7 REFERENCES


insight into the interaction of ADP-ribose with the PARP WWE domains. FEBS Lett. Nov 2; 586(21): 3858-64.


proteasomes. FEBS letters 457, 333-338.


