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Proteasome Regulation by ADP-Ribosylation

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SUMMARY

Protein degradation by the ubiquitin-proteasome system is central to cell homeostasis and survival. Defects in this process are associated with diseases such as cancer and neurodegenerative disorders. The 26S proteasome is a large protease complex that degrades ubiquitinated proteins. Here, we show that ADP-ribosylation promotes 26S proteasome activity in both *Drosophila* and human cells. We identify the ADP-ribosyltransferase tankyrase (TNKS) and the 19S assembly chaperones dp27 and dS5b as direct binding partners of the proteasome regulator PI31. TNKS-mediated ADP-ribosylation of PI31 drastically reduces its affinity for 20S proteasome α subunits to relieve 20S repression by PI31. Additionally, PI31 modification increases binding to and sequestration of dp27 and dS5b from 19S regulatory particles, promoting 26S assembly. Inhibition of TNKS by either RNAi or a small-molecule inhibitor, XAV939, blocks this process to reduce 26S assembly. These results unravel a mechanism of proteasome regulation that can be targeted with existing small-molecule inhibitors.

INTRODUCTION

Selective protein degradation plays a central role for the removal of misfolded and potentially toxic proteins, the control of cell-cycle progression, the regulation of gene expression, and changes in cell size and morphology (Baumeister et al., 1998; Demartino and Gillette, 2007; Finley, 2009; Glickman and Ciechanover, 2002; Hershko, 2005; Hershko and Ciechanover, 1998; Murata et al., 2009; Tanaka et al., 2012). Moreover, abnormal protein degradation is associated with a wide range of human diseases, such as cancer, muscle-wasting diseases, and neurodegenerative disorders (Glickman and Ciechanover, 2002; Goldberg, 2007; Hershko and Ciechanover, 1998). The selective degradation of most intracellular proteins is carried out by the ubiquitin-proteasome system (UPS) (Finley, 2009; Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998; Varshavsky, 2012). Proteins tagged with polyubiquitin chains are hydrolyzed into small peptides by the 26S proteasome in an energy-dependent manner (Baumeister et al., 1998; Besche et al., 2009b; Demartino and Gillette, 2007; Finley, 2009; Tanaka et al., 2012; Tomko and Hochstrasser, 2011).

The 26S proteasome is a large protease complex composed of a catalytic 20S subunit (also known as 20S core particle) and a 19S regulatory particle that caps one or both ends of the 20S proteasome (Baumeister et al., 1998; Besche et al., 2009b; Demartino and Gillette, 2007; Finley, 2009; Lander et al., 2012; Lasker et al., 2012; Murata et al., 2009; Tanaka et al., 2012; Tomko and Hochstrasser, 2011). The assembly and activity of the 26S proteasome is tightly regulated by a large number of loosely associated proteins that function as regulators or cofactors (Besche et al., 2009b; Finley, 2009; Tanaka et al., 2012; Tomko and Hochstrasser, 2011). One such factor is PI31, an evolutionarily conserved regulator of proteasome activity (Bader et al., 2011; Chu-Ping et al., 1992; McCutchen-Maloney et al., 2000; Zaiss et al., 1999). PI31 was initially identified on the basis of its ability to inhibit 20S proteasome activity in vitro (Chu-Ping et al., 1992; McCutchen-Maloney et al., 2000; Zaiss et al., 1999). However, PI31 can also activate the 26S proteasome in vitro, and mutational inactivation of the corresponding gene in *Drosophila* causes lethality-reduced proteasome activity and defects in protein degradation in vivo (Bader et al., 2011). Therefore, PI31 serves a crucial physiological function as an activator of 26S proteasome activity. The C terminus of PI31 contains a functionally important HbYX (hydrophobic residue-tyrosine-any amino acid) motif, which is commonly found in modulators of proteasome activity, such as Rpt base subunits of the 19S regulatory particle (Gillette et al., 2008; Rabl et al., 2008; Smith et al., 2007). This suggests that PI31 can bind to the 20S particle via its HbYX motif, and this may cause inhibition by hindering substrate access to the enzymatic core (Bader et al., 2011; McCutchen-Maloney et al., 2000). However, the precise molecular mechanism by which PI31 modulates proteasome activity remains unknown. Previous work also indicated that PI31 function is regulated in vivo in order to increase 26S proteasome activity under conditions where maximal proteolytic activity is required, for example, for the removal of most cellular proteins during the terminal differentiation of sperm (Bader et al., 2011). To gain further insight into the regulation of PI31 activity, we looked for previously unrecognized binding partners of this protein and identified the ADP-ribosyltransferase tankyrase (TNKS) as a direct interactor that modulates PI31 activity. TNKS-mediated ADP-ribosylation of PI31 is necessary for the ability of this protein to stimulate 26S proteasome function, and the

inhibition of TNKS reduces 26S proteasome activity in both *Drosophila* and mammalian cells. TNKS-mediated ADP-ribosylation of PI31 drastically reduces the affinity of this protein for binding to 20S proteasome α subunits and, thereby, relieves 20S repression by PI31. We also identified the 19S assembly chaperones dp27 and dS5b as binding partners of PI31. In this case, ADP-ribosylation of PI31 causes increased binding to dp27 and dS5b and sequestration of these assembly chaperones from 19S regulatory particles, which promotes 26S assembly. These results reveal an unexpected mechanism of proteasome regulation and define TNKS inhibitors as a distinct class of proteasome modulators that may have utility in the clinic.

RESULTS

dTNKS Interacts with DmPI31

To gain insight into the mechanism of PI31-mediated proteasome regulation, we looked for binding partners of this protein. For this purpose, we screened a *Drosophila* embryo complimentary DNA (cDNA) library by far-western blot analysis using P^{32}-labeled, HMK-tagged *Drosophila* PI31 (DmPI31) as a probe. This led to the identification of *Drosophila* tankyrase (dTNKS, CG4719) as a potential interacting protein (data not shown). dTNKS protein is 44% homologous to human tankyrase 1 and 2 (hTNKS1/2; Figure S1A available online). Next, we used coimmunoprecipitation (co-IP) experiments to investigate endogenous interactions between DmPI31 and dTNKS (Figure 1B). Extracts from wild-type (WT) embryos were immunoprecipitated with anti-DmPI31, and blots were probed with an antibody raised against dTNKS (Figures 1A and S1B). These experiments showed that dTNKS and DmPI31 form a complex in vivo (Figure 1B). We also coexpressed FLAG-tagged dTNKS and HA-tagged DmPI31 in human embryonic 293 (HEK293) cells to establish a cell-culture system for subsequent interaction studies (Figure 1C). Again, co-IP experiments with extracts from these cells showed the presence of a DmPI31:dTNKS complex (Figure 1C).

TNKS belongs to the poly(ADP-ribose) polymerase (PARP) superfamily (D'Amours et al., 1999; Hsiao and Smith, 2008; Smith et al., 1998). TNKSs play diverse roles in telomere maintenance, centrosome maturation, Wnt signaling, embryonic development, and the pathogenesis of Cherubism (Guettler et al., 2011; Hsiao and Smith, 2008; Huang et al., 2009; Levaot et al., 2011). TNKSs recruit and modify target proteins by ADP-ribosylation with the use of their Ankyrin (ANK) and PARP domains (Figure S1A) (Guettler et al., 2011; Hsiao and Smith, 2008; Huang et al., 2009; Levaot et al., 2011; Morrone et al., 2012; Smith et al., 1998). Unlike in mammals, where two isoforms of TNKS with partially redundant function are present, the *Drosophila* genome contains only one TNKS (Chiang et al., 2006, 2008; Hsiao et al., 2006; Hsiao and Smith, 2008; Yeh et al., 2009). In order to identify which region of dTNKS is responsible for interaction with DmPI31, we generated truncations of dTNKS that lacked either the ANK or SAM-PARP domains and tested them for binding to DmPI31 (Figures 1D and 1E). Whereas the SAM-PARP domains were dispensable for DmPI31 binding, deletion of the ANK domain in the $dTNKS^{\Delta ANK}$ mutant prevented the formation of the DmPI31:dTNKS complex (Figure 1E). This suggests that dTNKS binds DmPI31 via its ANK domain.

A hallmark of TNKS-binding partners is the presence of a canonical TNKS-binding motif (RxxGxGxE/D) (Guettler et al., 2011; Hsiao and Smith, 2008; Levaot et al., 2011). DmPI31 contains a good fit to this consensus motif at its N terminus (Figure 1F). To investigate the functional importance of this motif, we mutated this sequence by altering two amino acids (RG49/54AA) and tested binding to dTNKS. The mutant protein was no longer able to form a complex with dTNKS, indicating that this motif is required for interaction with dTNKS (Figure 1G, lane 2). To further characterize the DmPI31-dTNKS interaction, we generated DmPI31 mutants in which the C-terminal residues, including the HbYX motif, were either mutated or deleted (L210A, D211K, F241A and P243A, and ΔHbYX; Figure 1G, lanes 3–7). Whereas $DmPI31^{D211K}$ and $DmPI31^{P243A}$ mutants retained their ability to bind dTNKS, $DmPI31^{L210A}$ and $DmPI31^{F241A}$ were unable to form a complex with dTNKS (Figure 1G). Furthermore, deletion of the HbYX motif in DmPI31 also abrogated the interaction between DmPI31 and dTNKS (Figure 1G, lane 7). This suggests that dTNKS uses its ANK domain to bind DmPI31 and that both the N-terminal TNKS-binding motif and the C-terminal HbYX domain of DmPI31 are required for interaction with dTNKS.

dTNKS ADP-Ribosylates DmPI31

TNKSs modulate the activity of target proteins by ADP-ribosylation (Hsiao and Smith, 2008; Huang et al., 2009; Smith et al., 1998). Therefore, we investigated whether DmPI31 is a substrate for dTNKS-mediated ADP-ribosylation. First, we used western blot analysis with a monoclonal antibody recognizing poly (ADP-ribosylation) and found that DmPI31 is ADP-ribosylated in vivo (Figure 2A, lane 3). Next, we performed an in vitro ADP-ribosylation assay by incubating biotin-labeled NAD^+ and His-DmPI31 along with either FLAG-$dTNKS^{WT}$ or His-$dTNKS^{\Delta PARP}$ recombinant proteins (Figure 2B). These experiments demonstrated that dTNKS can directly ADP-ribosylate DmPI31 in vitro, and that this activity requires its PARP domain.

Next, we investigated the significance of TNKS-mediated ADP-ribosylation for PI31 function and proteasome regulation in *Drosophila* and, subsequently, in mammalian cells. For this purpose, we took advantage of XAV939, a small-molecule inhibitor of hTNKS1/2 (Huang et al., 2009). XAV939 was discovered in a screen for small molecules affecting the Wnt/β-catenin signaling pathway (Huang et al., 2009). XAV939 is a highly specific inhibitor of hTNKS1/2 (K_d = 0.09 μM) and has anticancer effects toward APC-deficient colorectal cancer cells (Huang et al., 2009). We tested the ability of XAV939 to inhibit dTNKS and found that it was able to inhibit the auto-ADP-ribosylation activity of dTNKS, but not PARP (Figures 2C and S2A) (Gibson and Kraus, 2012; Smith et al., 1998). Therefore, XAV939 is an effective and specific TNKS inhibitor in *Drosophila*.

TNKS Regulates Proteasome Activity by ADP-Ribosylation

Because DmPI31 is required for optimal proteasome activity in vivo and because dTNKS can posttranslationally modulate DmPI31, we explored the possibility that the inhibition of TNKS affects proteasome function. First, we examined the effect of the TNKS inhibitor XAV939 on 26S proteasome activity in

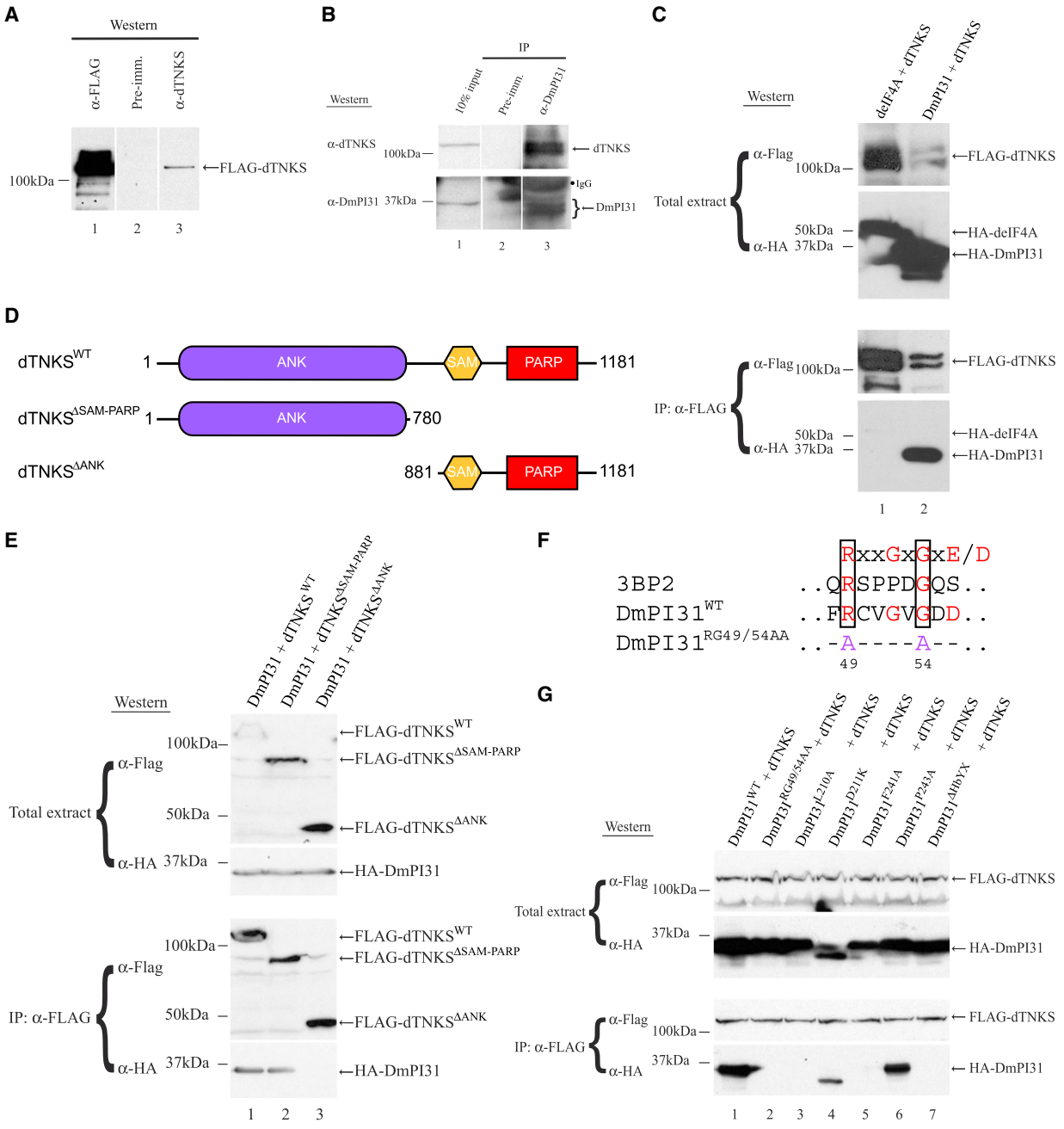

Figure 1. dTNKS, an Interacting Partner of DmPI31

(A) dTNKS antiserum detects purified dTNKS. Purified FLAG-tagged dTNKS is detected in a western blot with a dTNKS antiserum (lane 3), but not with preimmune serum (lane 2).

(B) In order to demonstrate that DmPI31 interacts with dTNKS in vivo, 0–2 hr *yw* (WT) embryo extract was used.

(C) A DmPI31:dTNKS complex can form in mammalian HEK293 cells. The anti-FLAG IP experiments were performed with extracts from HEK293 cells expressing FLAG-tagged dTNKS along with HA-tagged deIF4A (negative control) or DmPI31.

(D) A schematic representation of WT and mutant dTNKS with their respective domains. Numbers denote amino acid positions.

(E) dTNKS recruits DmPI31 through its ANK domain, as demonstrated via a co-IP experiment using HEK293 cell extracts expressing HA-tagged DmPI31 with FLAG-tagged dTNKS^{WT}, dTNKS$^{\Delta SAM\text{-}PARP}$, and dTNKS$^{\Delta ANK}$.

(F) Alignments of the TNKS-binding motif with those found in human 3BP2 (Guettler et al., 2011; Levaot et al., 2011) and DmPI31 reveal the presence of a putative motif in DmPI31. Numbers indicate the position of residues within DmPI31.

(G) dTNKS interacts with multiple DmPI31 surfaces, such as the putative DmPI31 TNKS-binding motif (RG49/54AA) and conserved C-terminal residues (D211 and P243), including the HbYX domain.

Also see Figure S1.

Drosophila. Extracts from 0–2 hr *yw* (WT) embryos were treated with different compounds and assayed for changes in proteasome activity (Figure 2E). Treatment with XAV939 significantly reduced 26S proteasome activity (Figure 2E). Although treatment with XAV939 did not cause complete inhibition of proteasome activity, as was observed for MG312, the effect was very similar to inactivation of DmPI31 (Bader et al., 2011). These results are consistent with a requirement of dTNKS for DmPI31-mediated proteasome activation. Next, we asked whether XAV939 could also inhibit proteasome activity in mammalian cells. For this purpose, we treated HEK293 cell extracts with XAV939 and measured 26S proteasome activity (Figure 2F). Again, we found that exposure to this compound decreased proteasome activity. Similar results were obtained with IWR-1, another TNKS inhibitor that is structurally unrelated to XAV939 (Figure S2B) (Chen et al., 2009; Narwal et al., 2012). This supports the conclusion that the observed decrease in proteasome activity is caused by TNKS inhibition and not an unrelated off-target effect of XAV939. To further corroborate this idea, we also used RNA interference (RNAi) to target TNKS in both *Drosophila* and mammalian cells. First, we expressed RNAi against dTNKS in the *Drosophila* retina in a background that was compromised for proteasome activity (Figure 3). Specifically, expression of temperature-sensitive dominant-negative mutant for the 20S proteasome β2 and β6 subunits caused a rough, reduced eye (Belote and Fortier, 2002). Expression of RNAi against dTNKS further enhanced this phenotype, similar to what was previously observed for reduction of DmPI31 function (Figures 3C–3E) (Bader et al., 2011). Therefore, downregulation of dTNKS reduces proteasome activity in vivo. Finally, we used small interfering RNA (siRNA) to knock down hTNKS1 and hTNKS2 in HEK293 cells (Figure 2D). Again, a significant reduction of 26S proteasome activities was observed (Figure 2G). Altogether, these results show that TNKS stimulates proteasome activity by ADP-ribosylation of PI31.

ADP-Ribosylation Blocks DmPI31 Inhibition of 20S Proteasomes

Best known for its role in DNA damage, ADP-ribosylation is a transient posttranslational modification that can drastically alter the physical properties of target proteins (Chambers et al., 2012; Gagné et al., 2006; Gibson and Kraus, 2012; Wang et al., 2009). In order to understand the biochemical consequences of TNKS-mediated ADP-ribosylation of PI31, we investigated whether this modification affects the binding properties of this protein. In mammalian cells, PI31 can bind to 20S proteasomes (McCutchen-Maloney et al., 2000; Zaiss et al., 1999). Likewise, we found that DmPI31 can bind to 20S proteasomes in *Drosophila* (Figure 4A). In particular, DmPI31 bound selectively to several α subunits (α1, α3, α4, and α6, but not α2 and α5) (Figure 4A). Next, we used co-IP experiments to evaluate the role of ADP-ribosylation on the DmPI31:α4 complex. Whereas unmodified His-PI31 strongly interacted with α4, binding of the ADP-ribosylated version was significantly diminished (Figure 4B). Consistent with the observed change in DmPI31 affinity for α subunits, we found that ADP-ribosylation interfered with the ability of PI31 to inhibit 20S proteasome particles (Figure 4C). These results demonstrate that ADP-ribosylation blocks the binding of DmPI31 to 20S subunits and relieves repression of their proteolytic activity by this protein.

dTNKS and DmPI31 Actively Participates in Proteasome Assembly

Given that TNKS-mediated ADP-ribosylation of PI31 affects both 20S and 26S proteasome activities, we investigated a possible role of TNKS and PI31 in 26S proteasome assembly. For this, we performed Superose 6 gel filtration (fast protein liquid chromatography [FPLC]) assays and native gel analyses to study the effect of TNKS inhibitor XAV939 on proteasome assembly. *Drosophila* embryos are an ideal system to study effects on 26S assembly because ~65% of proteasomes exist as 20S particles (Nickell et al., 2007). XAV939 treatment of *Drosophila* embryo extracts led to a significant shift in Rpt bands toward lighter fractions when compared to controls, and it prevented the formation of 26S proteasomes when analyzed on native gels (Figures 5A, 5B, S3A, and S3B). This indicates that TNKS activity promotes 26S proteasome assembly.

Next, we examined the effect of PI31 on 26S proteasome assembly. Again, we used gel filtration assays to analyze embryo extracts supplemented with high concentrations of His-DmPI31 recombinant protein (Figure 5C). Again, the addition of His-DmPI31 caused a significant change in the proteasome architectural landscape by redistributing Rpt3 bands (Figure 5C). We also observed an increase in Rpt3 monomers (Figure 5C, top panel; fractions 20–23). These effects were seen for WT DmPI31, but not for a mutant lacking the HbYX motif (Figure 5C). The increase in Rpt3 monomers can be explained by the ability of PI31 to bind 19S assembly chaperones (see below) that are known to stabilize the assembly of both the 19S regulatory particle and the 26S proteasome (Funakoshi et al., 2009; Kaneko et al., 2009; Le Tallec et al., 2009; Park et al., 2009; Roelofs et al., 2009; Saeki et al., 2009). Collectively, these results show that both TNKS activity and PI31 can modulate 26S proteasome assembly, and this work suggests a role of PI31 in promoting 19S stability.

19S Assembly Chaperones Interact with DmPI31

To better understand the role of DmPI31 in proteasome assembly, we looked for additional DmPI31-binding partners. In the same screen that revealed dTNKS as a DmPI31 interactor, we discovered two other proteasome-associated proteins—*Drosophila* p27 (dp27, CG9588) and *Drosophila* S5b (dS5b, CG12096). p27 and S5b play a known role as 19S assembly chaperones and, along with Rpn14 and Nas6, bind to 19S Rpt subunits in order to foster orderly proteasome assembly (Funakoshi et al., 2009; Kaneko et al., 2009; Le Tallec et al., 2009; Park et al., 2009; Roelofs et al., 2009; Saeki et al., 2009). As in yeast and humans, the *Drosophila* homologs of the 19S assembly chaperones (dp27, 41% homologous; dS5b, 23.3% homologous; Figures 6B and S5A) are essential genes and are required for organismal viability (Figures 3F–3L, S4A, and S4B). Immunoprecipitation (IP) experiments with anti-DmPI31 using *Drosophila* embryo extracts followed by western blot analysis with anti-dp27 reveal that DmPI31 binds dp27 in vivo (Figures 6A and S4C). To identify DmPI31 residues involved in dp27 and dS5b-binding, we used point mutants previously employed to map

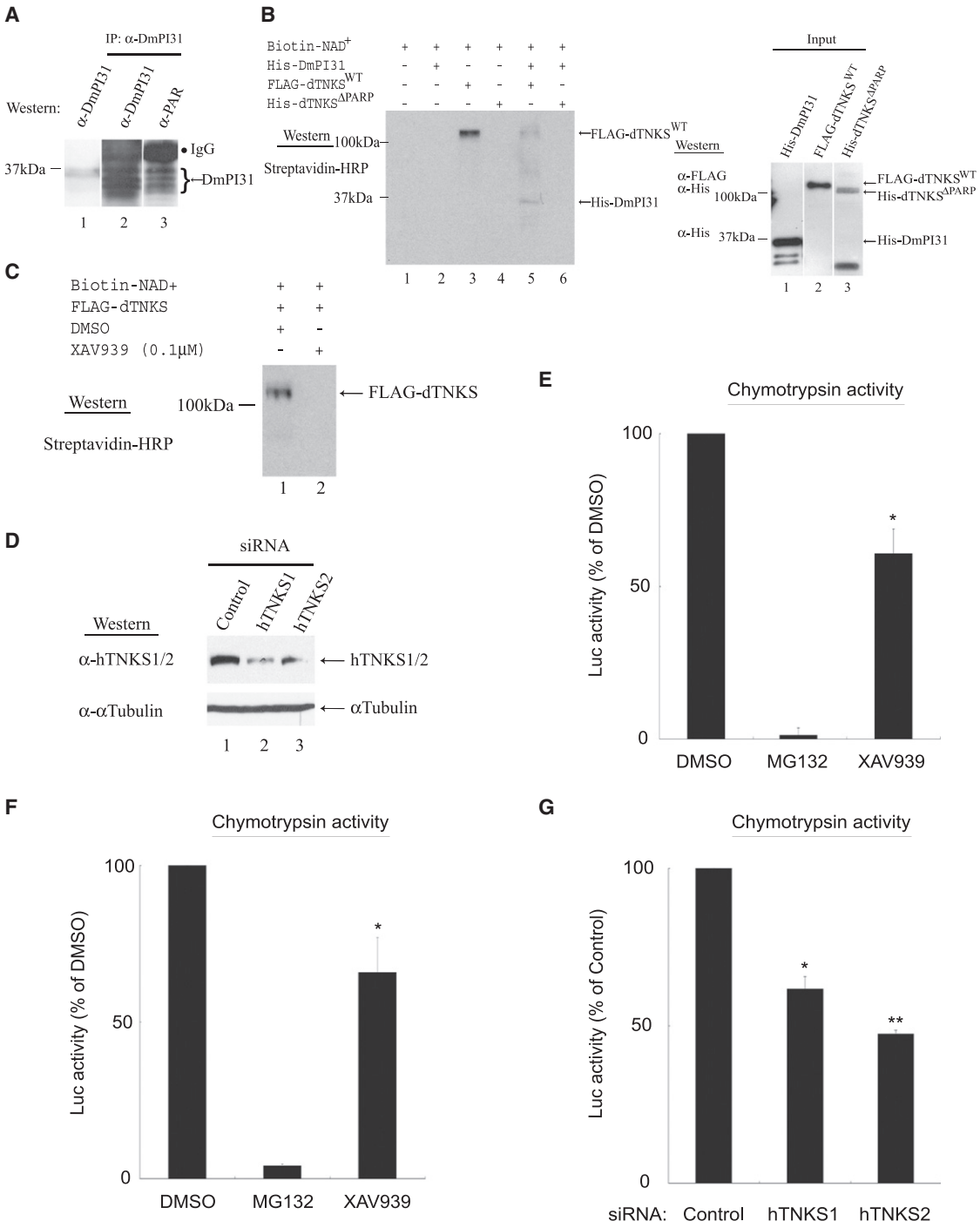

Figure 2. dTNKS Posttranslationally Modifies PI31 via ADP-Ribosylation to Regulate 26S Proteasome Activity

(A) DmPI31 is poly(ADP-ribosylated) in vivo. Endogenous DmPI31 ADP-ribosylation was detected with 0–2 hr WT embryo extracts immunoprecipitated with anti-DmPI31. Eluted proteins were analyzed by western blotting with an anti-poly(ADP-ribose) (PAR) antibody.

(B) dTNKS directly ADP-ribosylates DmPI31 in vitro, and this is dependent on its PARP domain. Samples containing biotin-NAD^+, His-DmPI31, and FLAG-$dTNKS^{WT}$ or His-$dTNKS^{\Delta PARP}$ were analyzed for ADP-ribosylation by western blot analysis. Input proteins were also analyzed by western blotting (right panel).

(C and D) Tools used to investigate the role of TNKS in 26S proteasome regulation. (C) XAV939 inhibits *Drosophila* TNKS (dTNKS) in vitro, indicating that the activity of this drug is conserved across species. (D) siRNAs targeting hTNKS1 and hTNKS2 efficiently knocked down expressions in HEK293 cells. α-tubulin was used as a loading control.

(E and F) TNKS inhibition by XAV939 reduces proteasome activity in *Drosophila* embryo (E) and HEK293 cell (F) extracts.

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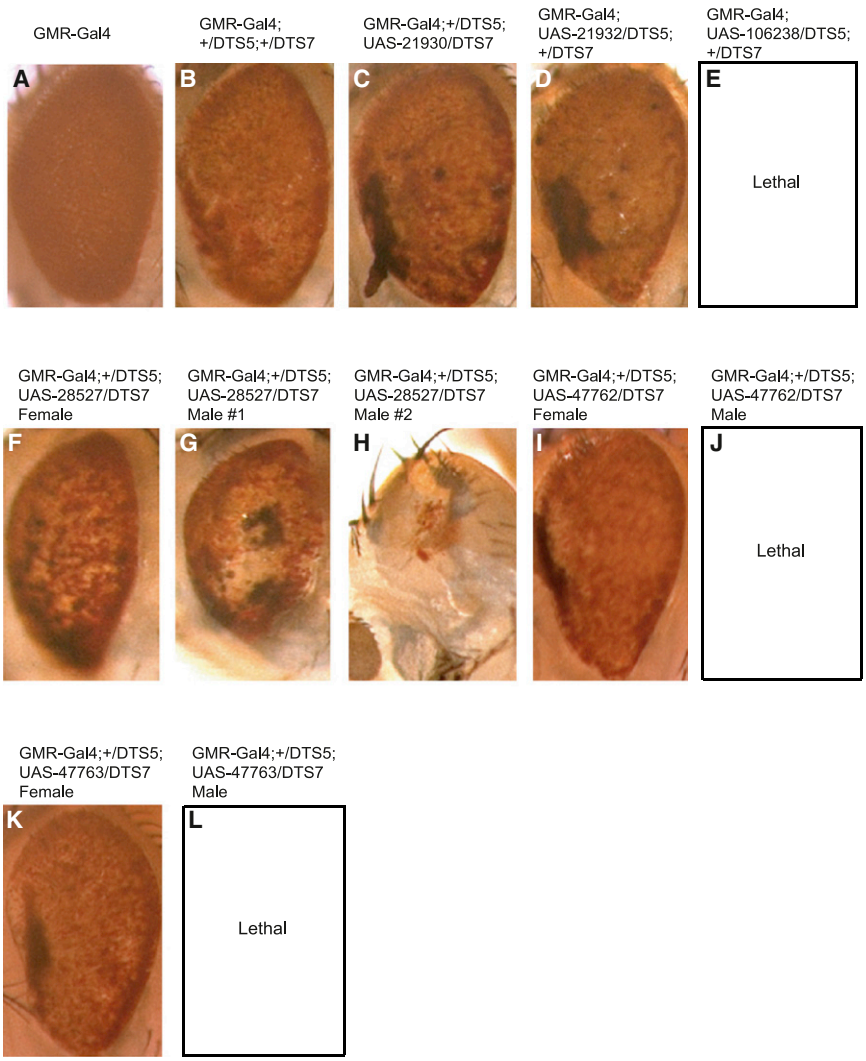

Figure 3. dTNKS and dp27 RNAi Enhance Compromised Proteasome Activity

To test the role of dTNKS and dp27 in modulating proteasome activity in vivo, we expressed RNAi against dTNKS (21930, 21932, and 106238; C–E) and dp27 (28527, 47762, and 47763; F–L) in the background of dominant-negative temperature-sensitive mutants (UAS-DTS5 and UAS-DTS7) at 29°C. As expected, downregulation of these factors resulted in a significant enhancement of the mutant rough reduced eye phenotype (compare B to C–L) and, in some dp27 RNAi lines, caused male lethality. These observations are indicative of reduced proteasome activities in these tissues. GMR-Gal4 expression caused no discernable eye defects (A). Orientation of eyes are anterior left and dorsal up.

the DmPI31:dTNKS interaction (Figure 1G). Although mutants defective for TNKS binding (RG49/54AA) failed to destabilize the DmPI31:assembly chaperone complex, mutating the conserved C terminus of DmPI31 (L210, F241, and the HbYX motif) disrupted these interactions (Figures 6C and S5B). These results show that the C-terminal HbYX motif is required for the formation of a complex between DmPI31 and the assembly chaperones dp27 and dS5b.

Despite their structural differences, 19S assembly chaperones use a common mechanism to bind to 19S Rpt subunits (Barrault et al., 2012; Funakoshi et al., 2009; Lee et al., 2011; Park et al., 2009; Roelofs et al., 2009; Saeki et al., 2009; Takagi et al., 2012). Because dp27 and dS5b both interact with DmPI31 C terminus, we performed protein sequence alignments of dp27, dS5b, and dRpn14. This identified three putative DmPI31-binding motifs (motifs I–III; Figure 6D). In order to test the functional relevance of these motifs, we mutated these sites and examined the consequences for DmPI31 interaction (Figures 6E and S5C). Point mutations that replaced key residues in motifs I and III of dp27 and dS5b (dp27, E9A and R187A; dS5b, E44A, and R263A) failed to recruit DmPI31, indicating a requirement of these motifs for interaction with DmPI31 (Figures 6E and S5C). These results identify dp27 and dS5b as interacting partners of DmPI31.

ADP-Ribosylation Promotes 26S Proteasome Assembly by Increasing the Affinity of DmPI31 for Assembly Chaperones

Given that ADP-ribosylation of PI31 modulates 20S proteasome activity by altering its affinity to α subunits (Figure 4), we wondered if this posttranslational modification may also affect interaction with the 19S assembly chaperones p27 and S5b. First, to examine this possibility, we performed an anti-FLAG IP experiment using HEK293 cell extracts standardized for FLAG-dp27 and HA-dRpt5 expression followed by rigorous washing and the addition of incremental amounts of in vitro modified DmPI31. The effects of ADP-ribosylated DmPI31 on the stability of the dp27:dRpt5 and dS5b:dRpt2 complexes were visualized via western blot (Figures 7A and S6A). Countering its effect on α4 subunit, ADP-ribosylation considerably increased the ability of DmPI31 to recruit the assembly chaperones when compared to its unmodified version, sequestering them away from the Rpt base subunits in a dose-dependent manner (compare Figures 7A and S6A to Figures 7B and S6B, respectively). These results,

(G) Reduced hTNKS1 and hTNKS2 levels inhibit 26S proteasome activity.

In (E)–(G), data are presented as mean ± SD from three independent experiments. Values obtained for DMSO (E and F) and control siRNA (G) were set as 100%. (E) *, p = 0.01; (F) *, p = 0.05; (G) *, p = 0.04 and **, p = 0.006. Statistical analysis was performed with a two-tailed paired t test.

Also see Figure S2.

along with those presented earlier, indicate that ADP-ribosylation directly modifies DmPI31 affinity for proteasome-associated proteins.

Evidence for a role of PI31 as an activator of the 26S proteasome was previously based on the requirement of this protein for normal proteasome activity in vivo and the ability of this protein to stimulate 26S proteasome activity in vitro (Bader et al., 2011). Because modification by ADP-ribosylation differentially affects the interaction of DmPI31 with different proteasome proteins, we investigated the consequences of PI31modification on 26S proteasome activity in vitro. ADP-ribosylated DmPI31 was significantly more potent in stimulating the chymotrypsin-like activity of purified 26S proteasomes in vitro (Figure 7C). Furthermore, when modified DmPI31 was incubated with purified 20S and 19S particles and analyzed on native gels, we saw that ADP-ribosylation increased the ability of DmPI31 to promote de novo 26S assembly in vitro (Figure 7D). Finally, the addition of ADP-ribosylated DmPI31 to extracts pretreated with XAV939 reversed the inhibitory effect of this compound on 26S proteasome assembly and was even able to further increase 26S proteasome assembly (Figure 7E). Also, we found that surplus PI31 can overcome the effects of XAV939, albeit less efficiently than its modified counterpart. This is consistent with our observation that unmodified PI31 can bind and sequester 19S assembly chaperones (Figure S6B). Altogether, our data show that dTNKS-dependent ADP-ribosylation of DmPI31 stimulates 26S proteasome activity by promoting 26S assembly.

DISCUSSION

It is becoming increasingly clear that the proteasome, often thought to be a constitutively active protease complex, is dynamically regulated in order to meet the changing proteolytic needs of a cell (Demartino and Gillette, 2007; Glickman and Ciechanover, 2002; Hershko and Ciechanover, 1998). Although the polyubiquitination of substrates is central in selecting specific proteins for degradation, additional mechanisms must exist to account for the plasticity of proteasome activity (Crosas et al., 2006; Demartino and Gillette, 2007; Hanna et al., 2007; Kurucz et al., 2002; Murata et al., 2009; Peth et al., 2009, 2013, 2010; Princiotta et al., 2001). Here, we identified TNKS as a regulator of proteasome activity in both *Drosophila* and mammalian cells. Specifically, we show that TNKS-mediated ADP-ribosylation of PI31 alters the affinity of this protein for 20S proteasome α subunits and 19S assembly chaperones and, thereby, stimulates 26S proteasome assembly. Our results support a model in which TNKS activates an evolutionarily conserved proteasome-regulatory protein, PI31, by ADP-ribosylation (Figure 7F). PI31 is physiologically required for optimal 26S proteasome activity in vivo, and inactivation of the corresponding gene in *Drosophila* causes reduced protein breakdown and organismal lethality (Bader et al., 2011). Inhibition of TNKS by either RNAi or with a specific small-molecule inhibitor, XAV939, blocked ADP-ribosylation of PI31 and impaired 26S proteasome activity in a manner similar to the inactivation of PI31. It was suggested that PI31 may act as a modulator of proteasome assembly by reversibly associating with 20S proteasome (Besche et al., 2009a; McCutchen-Maloney et al., 2000;

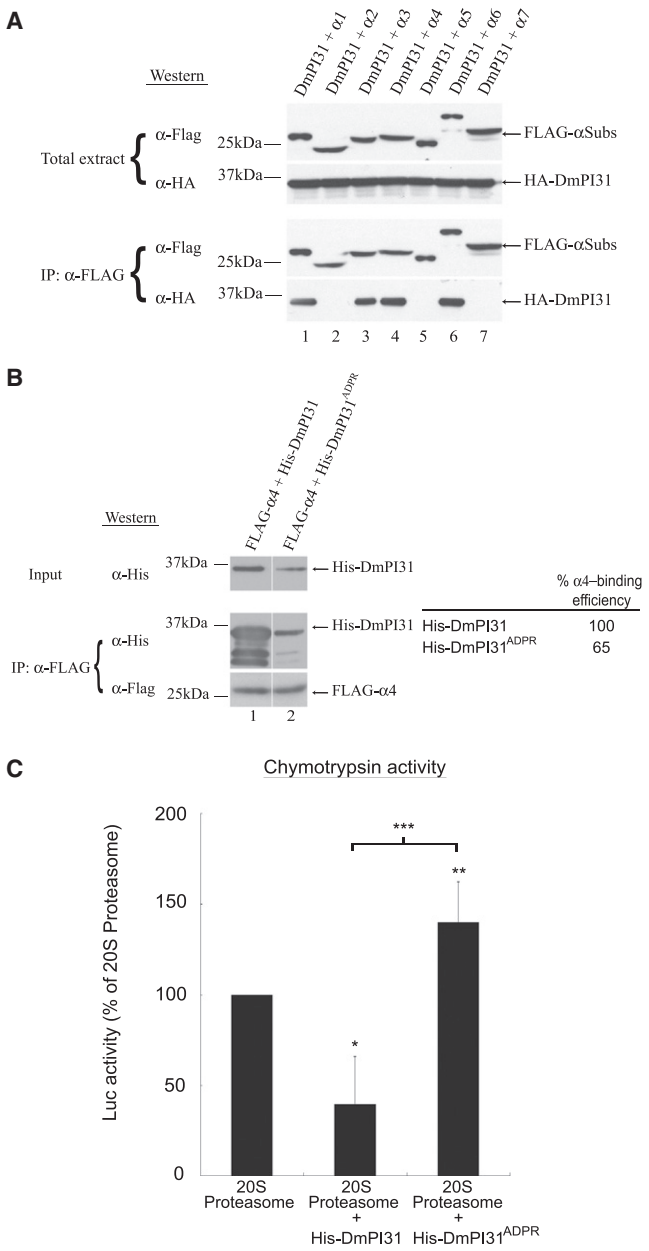

Figure 4. ADP-Ribosylation Relieves 20S Inhibition by PI31

(A) DmPI31 interacts with 20S proteasomes via select α subunits; i.e., α1, α3, α4, and α6.

(B) ADP-ribosylation significantly decreases the affinity of DmPI31 for α4. FLAG-α4-expressing HEK293 cell extract was incubated with modified DmPI31 (His-DmPI31^{ADPR}) to test its effect on the α4:DmPI31 complex. Chemoluminescence was quantitated with ImageQuant LAS 4000 (GE Healthcare). α4 binding is reported as a relative amount of immunoprecipitated His-DmPI31 in comparison to the level of input His-DmPI31.

(C) Modification of DmPI31 relieves its inhibitory effect on 20S proteasomes. Purified bovine 20S proteasomes (0.1 μg) were incubated with His-DmPI31 and His-DmPI31^{ADPR} proteins (2 μg each), and chymotrypsin-like proteasome activity was measured. Data are presented as mean ± SD from three independent experiments. The value obtained for 20S proteasome was set as 100%. *, p = 0.04; **, p = 0.6; and ***, p = 0.045. Statistical analysis was performed with a two-tailed paired t test.

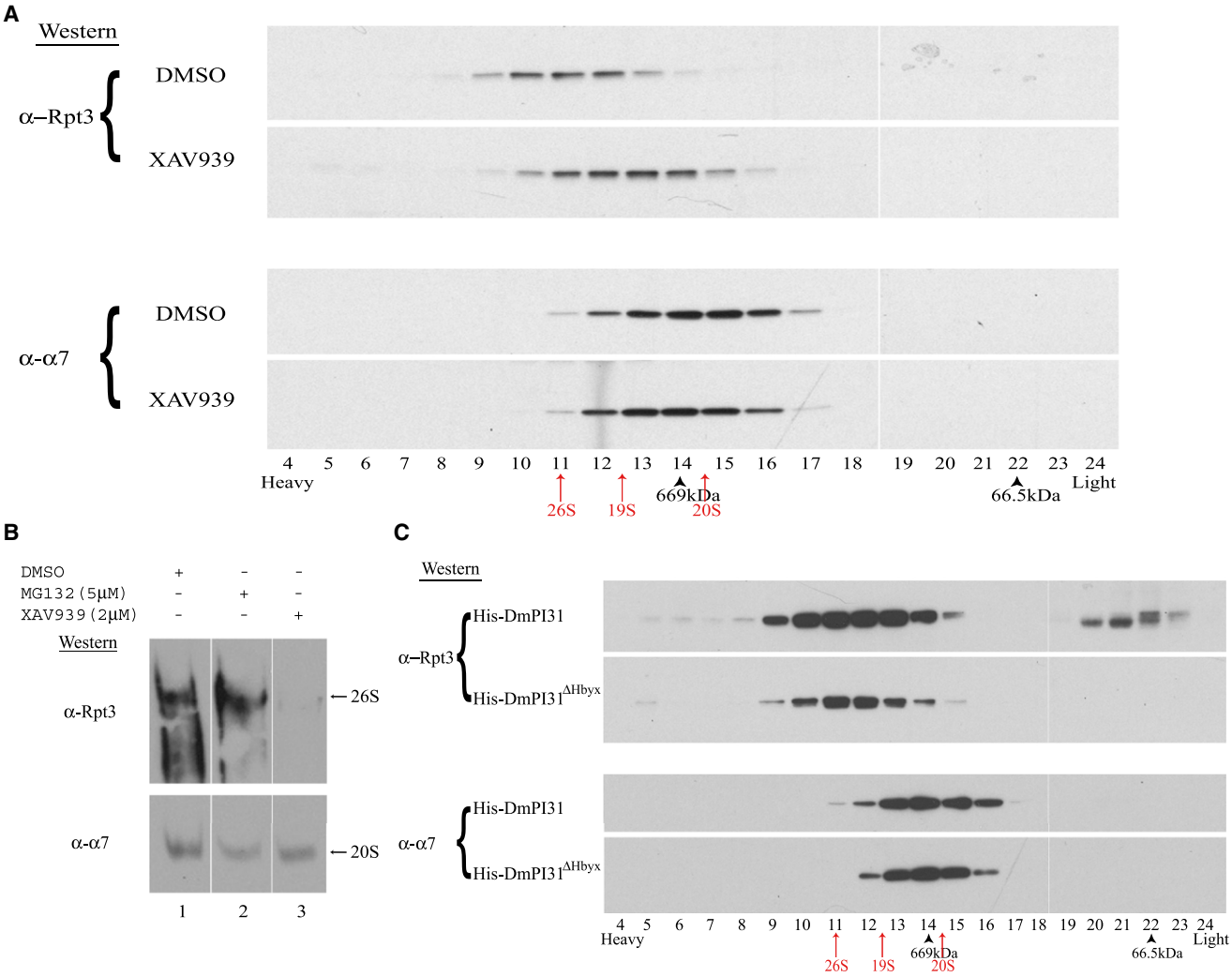

Figure 5. TNKS and PI31 Regulate Proteasome Assembly

(A) Inhibition of dTNKS by XAV939 led to a drastic shift of the proteasome profile to "lighter" fractions, indicative of reduced amounts of 26S particles. To assess the effect of TNKS activity on 26S assembly, we subjected embryo extracts treated with XAV939 to Superose 6 gel filtration chromatography.

(B) TNKS inhibition severely affects 26S proteasome assembly. The effect of XAV939 on proteasome assembly in embryo extracts was assessed by native gel analysis.

(C) Similar to XAV939, surplus DmPI31 dramatically affected proteasome assembly. Furthermore, it also caused an increase in levels of Rpt3 monomers (fractions 20–23), as visualized by gel filtration chromatography.

In (A)–(C), western blot analyses were performed to detect the presence of the proteasomal subunits Rpt3 (a 19S component that can be used to identify 26S) and α7 (to identify 20S). In (A) and (C), arrows and arrowheads indicate the position of proteasome components (red arrows; 26S, 19S, and 20S) and standards (black arrowheads; 660 kDa Thyroglobulin and 66.5 kDa BSA), respectively.

Also see Figure S3.

Tai et al., 2010; Tanahashi et al., 1999; Zaiss et al., 1999, 2002). In support of this idea, we show that PI31 modification by TNKS facilitates the assembly of the 26S proteasome by acting at two crucial stages (Figure 7F). First, TNKS-mediated ADP-ribosylation decreases the affinity of PI31 for α4 subunits of the 20S particle. This dislodges PI31 from 20S proteasomes and prevents PI31 from inhibiting 20S activity. Once free, modified PI31 competes with Rpt subunits to sequester the assembly chaperones p27 and S5b away from 19S regulatory particles, thereby promoting the capping of 20S with 19S particles. This is consistent with previous observations where the overexpression of p27, S5b, and Rpn14 (PAAF1) proteins negatively affected proteasome assembly (Kaneko et al., 2009; Lassot et al., 2007; Park et al., 2005; Shim et al., 2012). By acting on both 20S particles and assembly chaperones, PI31 appears to function as a central regulator of 26S proteasome assembly (Figure 7F). Significantly, this process can be disrupted with existing small-molecule compounds; TNKS inhibitors represent a distinct class of compounds that inhibit 26S activity by interfering with proteasome assembly.

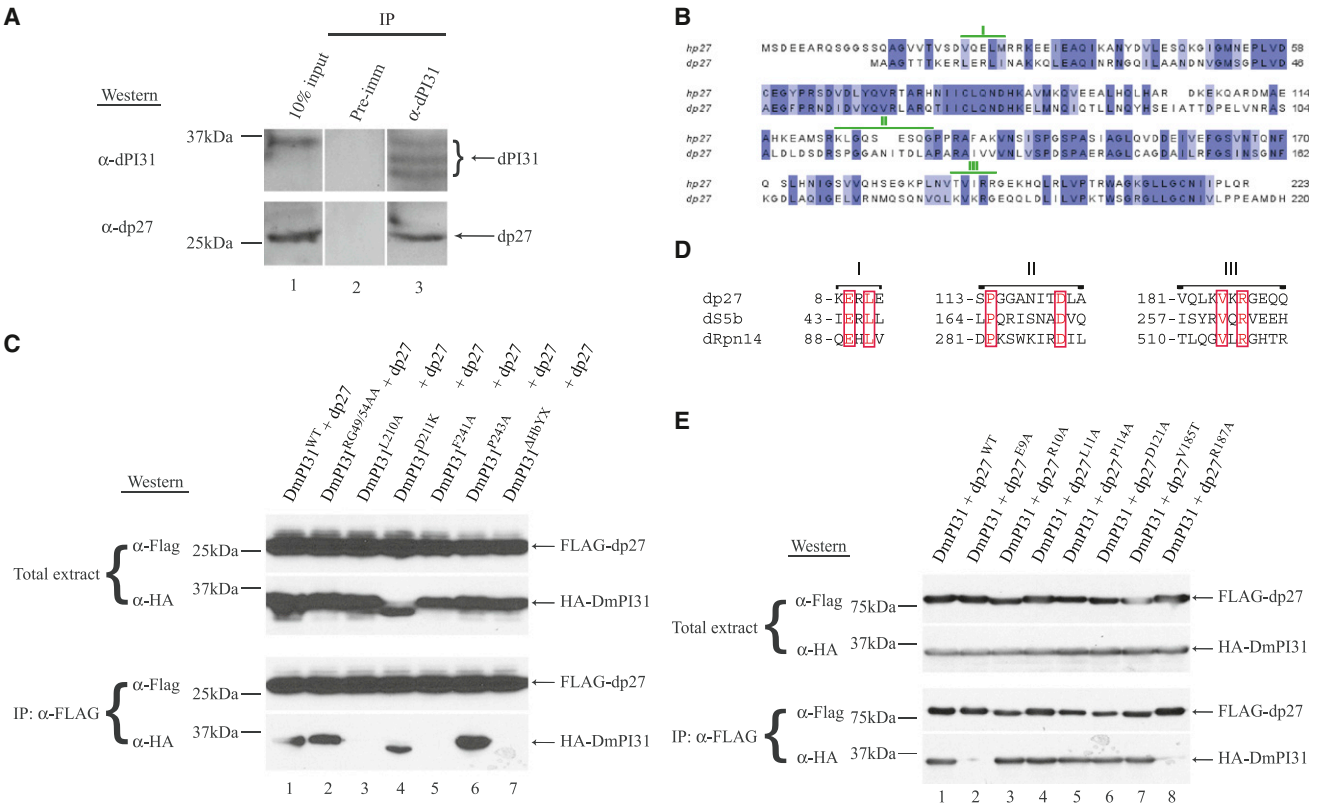

Figure 6. The 19S Assembly Chaperone dp27 Interacts with DmPI31

(A) Endogenous dp27:DmPI31 interaction was detected in an anti-DmPI31 IP experiment using embryo extracts.

(B) Sequence alignment of p27 from human (hp27) and *D. melanogaster* (dp27). dp27 is 41% homologous to hp27. Identical amino acids are colored in violet and the DmPI31-binding motifs (I-III; refer to D) are denoted in green.

(C) DmPI31 recruits dp27 via its C-terminal residues; i.e., L210, F241, and the HbYX domain. Similar observations were made for TNKS (Figure 1G).

(D) dp27, dS5b, and dRpn14 protein sequence alignments reveal three putative DmPI31-binding motifs (I–III) with unique conserved residues (shown in red boxes). Numbers denote N-terminal end amino acid positions of each motif in respective proteins.

(E) Multiple dp27 surfaces (motifs I [E9A] and III [R187A]) make contact with DmPI31 to stabilize the complex.

Also see Figures S4 and S5.

PI31 is required for normal proteasome activity in vivo, and it can also stimulate the activity of purified 26S proteasome in vitro (Bader et al., 2011). In order to explain the latter finding, we invoke the possibility that purified 26S proteasomes used in these experiments contain associated proteins, including 19S assembly chaperones. This is consistent with observations showing p27 and S5b in association with the purified 26S proteasome (Besche et al., 2009a; Deveraux et al., 1995; Gomes et al., 2006; Tai et al., 2010; Watanabe et al., 1998). Furthermore, unmodified PI31 can still bind to assembly chaperones, albeit with much lower efficiency (Figure S6B). Altogether, these results suggest that PI31 stimulates proteasome activity in vitro by increasing 26S particles.

The rate of intracellular protein degradation is dramatically affected by various signals, including the metabolic state of the cell (Glickman and Ciechanover, 2002). For example, caloric restriction can cause a severe reduction of skeletal muscles, and humans loose a large fraction of their muscle mass during aging. Although this appears to have evolved as a protective mechanism to cope with food shortage, muscle atrophy can be a highly debilitating process that also occurs in various diseases, such as cancer cachexia, AIDS, renal failure, and neurodegenerative diseases. In all these cases, proteasome activity is responsible for protein breakdown and cellular atrophy, but a direct connection between metabolism and proteasome activity has not yet been established. Given that NAD^+ is the source of the ADP-ribose group, proteasome regulation by TNKS provides a potential link between cell metabolism and the regulation of protein degradation (Gibson and Kraus, 2012). Because ADP-ribosylation has a very short half-life (1–2 min), it is ideally suited to rapidly signal transient changes in cell physiology (Chambers et al., 2012; Gibson and Kraus, 2012; Lindahl et al., 1995; Wang et al., 2009). We speculate that TNKS-mediated activation of PI31 may serve to stimulate proteasome activity when cells need to dynamically boost their proteolytic capacity in order to meet changing demands, such as during cellular remodeling, upon stress conditions, and during caloric restriction. It is also possible that the activation of PI31 occurs only in specific

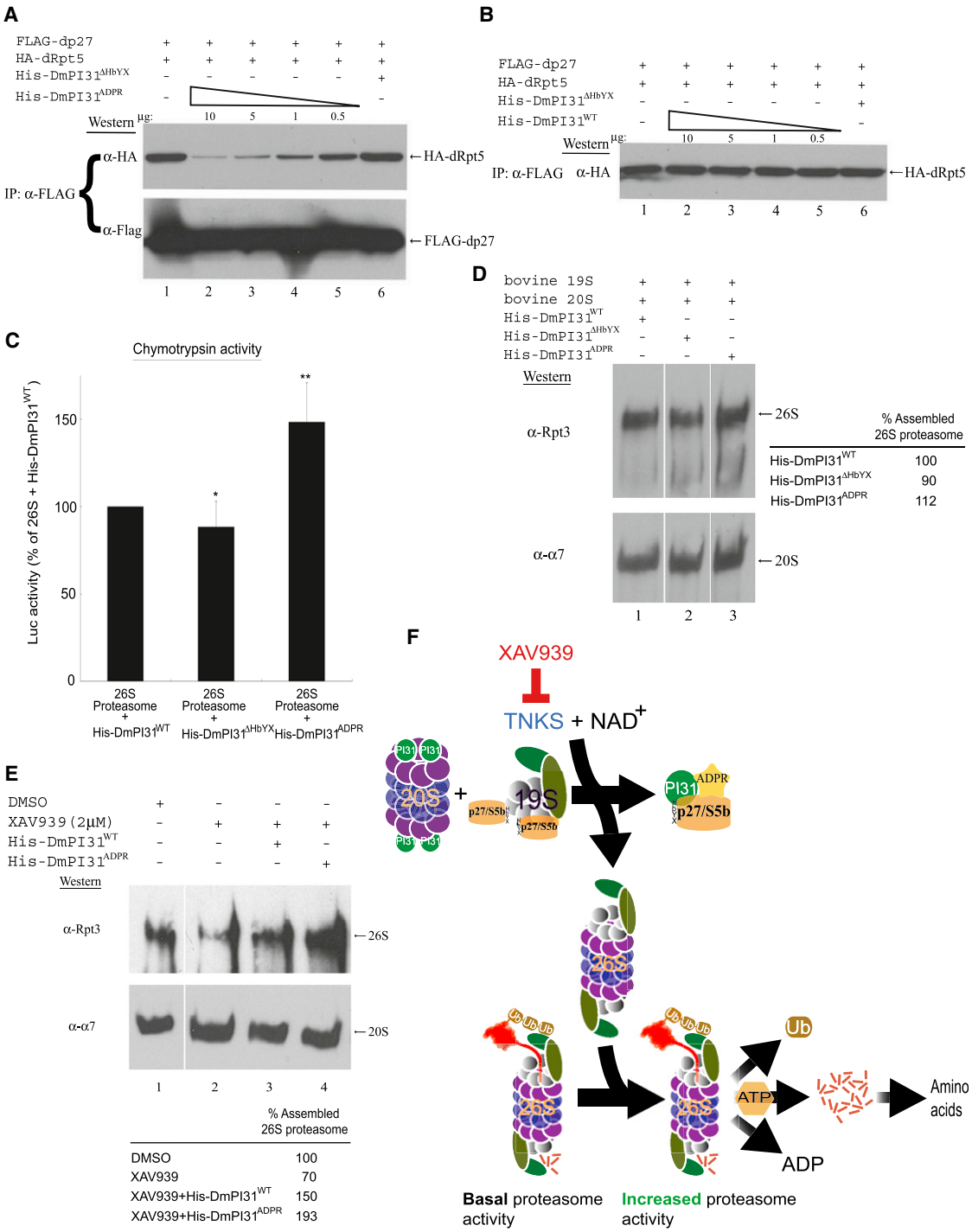

Figure 7. ADP-Ribosylation Increases DmPI31 Affinity for dp27 to Regulate Proteasome Assembly and a Model for Proteasome Regulation by ADP-Ribosylation

(A and B) ADP-ribosylation increased the affinity of DmPI31 for dp27, allowing it to compete with dRpt5 for chaperones in a dose-dependent manner. Anti-FLAG IP with HEK293 cell extracts standardized for FLAG-dp27 and HA-dRpt5 expressions were supplemented with in vitro modified (A) and unmodified (B) DmPI31 in order to assess their ability to compete with dRpt5 for dp27.

(C) ADP-ribosylated DmPI31 activated 26S proteasome activity in vitro to levels beyond those seen for its unmodified control. Purified bovine 26S proteasome (0.1 μg) was incubated with His-DmPI31, His-DmPI31$^{\Delta HbYX}$, and His-DmPI31^{ADPR} (2 μg each), and chymotrypsin-like proteasome activity was assayed. Data are presented as mean ± SD from three independent experiments. The value obtained for 26S + His-DmPI31^{WT} was set as 100%. *, p = 0.46; **, p = 0.007. Statistical analysis was performed with a two-tailed paired t test.

(legend continued on next page)

subcellular compartments; for example, within the nucleus, in specific neuronal processes undergoing pruning, or in synapses during remodeling. In this way, it may be possible to locally fine-tune the assembly and activity of 26S proteasomes to meet a cell's changing needs for controlled proteolysis.

The proteasome is a validated drug target for cancer therapy and bortezomib (VELCADE), which inhibits the chymotrypsin-like activity of the proteasome, is approved in the U.S. for the treatment of multiple myeloma and mantle cell lymphoma (Goldberg, 2012; Kisselev et al., 2012; Raab et al., 2009). Problems associated with bortezomib include notable side effects, such as peripheral neuropathy and drug resistance (Goldberg, 2012; Kisselev et al., 2012; Raab et al., 2009). Therefore, drugs that inhibit proteasome activity by a separate mechanism may have considerable clinical value. Our findings suggest that TNKS inhibitors, such as XAV939 and IWR-1, may be useful for the treatment of multiple myeloma, mantle cell lymphoma, and other cancers sensitive to proteasome inhibition. Consistent with this idea, we have preliminary evidence that XAV939 can block the growth of multiple myeloma cells (Figure S6C). Besides inhibiting proteasome activity by an entirely distinct mechanism, TNKS inhibitors are expected to only block maximal activation of 26S function, but not the basal activity of the proteasome, and, hence, may have fewer side effects (Figure 7F). Interestingly, XAV939 has already been shown to be effective against colorectal cancer cells and is thought to antagonize Wnt signaling by preventing the degradation of axin (Huang et al., 2009). However, the contribution of proteasome inhibition in this paradigm has not yet been determined. Proteasome regulation by tankyrase-mediated ADP-ribosylation provides an unexpected mechanism for the regulation of protein degradation that can be targeted with small-molecule inhibitors.

EXPERIMENTAL PROCEDURES

Recombinant Protein Purification and Far-Western Screening of the cDNA library

For the purification of His-DmPI31, His-DmPI31$^{\Delta HbYX}$, His-HMK-DmPI31, and His-dTNKS$^{\Delta PARP}$ fusion proteins, *E.coli* BL21 (Invitrogen) was transformed with the pET28-His-DmPI31, pET28-His-DmPI31$^{\Delta HbYX}$, pET101-His-HMK-DmPI31, and pET101-His-dTNKS$^{\Delta PARP}$ constructs. After a 2 hr induction at 37°C with 0.1 mM IPTG, the fusion proteins were purified on a TALON Metal Affinity Resin (BD Biosciences) according to the manufacturer's instructions. For the purification of FLAG-dTNKS and FLAG-dPARP, HEK293 cell extracts expressing the pcDNA3.2-FLAG-dTNKS and pcDNA3.2-FLAG-dPARP constructs were incubated with ANTI- FLAG M2 Affinity Gel (Sigma-Aldrich) for 3 hr and purified with FLAG peptide (Sigma-Aldrich) according to the manufacturer's instructions. Far-western screening of the cDNA library was performed as previously described (Pause et al., 1994) with the *Drosophila* λ cDNA Library (Stratagene).

Cell Culture and Growth Assay

Cationic lipid reagent (20 μl of Lipofectamine 2000; Invitrogen) was diluted in serum-free media (Opti-MEM; Invitrogen) for transfection in HEK293 cells (100 mm dish). Following a 5 hr incubation, the medium was replaced with Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Transfected cells were harvested in PBS 48 hr following the addition of serum-containing media. The cells were then lysed by repeated freeze/thaw cycles in 600 μl of lysis buffer (20 mM HEPES-KOH [pH 7.6], 200 mM KCl, 0.5mM EDTA, 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail [Complete; Roche]) that contained RNase A (50 μg/ml; Sigma-Aldrich). Cell debris was pelleted by centrifugation, and the protein concentration in the supernatant was determined with the Bio-Rad assay. The siRNAs purchased from Invitrogen (sequences shown in Table S1) were transfected in HEK293 cells at a final concentration of 50 nM/100 mm dishes with the use of Lipofectamine 2000. Effects of drug treatment on U266 multiple myeloma cell growth were performed with 0.1 μM of bortezomib and 5 μM of XAV939 in low-FBS (0.5%) RPMI media and 96-well plates seeded with ~8,000 cells per well. Samples were assayed for growth and viability with PrestoBlue Cell Viability Reagent (Invitrogen) in a Spectramax M2 reader (Molecular Devices) per the manufacturer's instructions.

Coimmunoprecipitation

For co-IP, HEK293 cell extract (200 μl; 6–10μg/μl) was brought up to 1 ml with the lysis buffer and precleared for 1 hr at 4°C with 25 μl of Protein A Sepharose (GE Healthcare). The supernatant was immunoprecipitated for 1 hr at 4°C with 25 μl of ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich). The resin was washed twice with lysis buffer and once with lysis buffer containing 300 mM KCl. Immunoprecipitates were eluted in 3X red sample buffer (New England Biolabs). For anti-HA and anti-DmPI31 IPs, 25 μl of Protein A Sepharose was preincubated for 2 hr with anti-HA (3 μl) and anti-DmPI31 (5 μl). The resins were washed three times with the lysis buffer prior to IP, as described above. Embryo extracts were used at a concentration of 5 μg/μl.

Proteasome Activity Assay

For proteasome activity assays, 50 μg of 0–2 hr WT embryo or 3 μg of HEK293 cell extracts in PIPES buffer (50 mM PIPES, 1 mM $MgCl_2$, 50 mM NaCl, 2 mM EGTA, and 2 mM ATP) were programmed with 1% DMSO (Sigma-Aldrich), 5 μM MG132 (Calbiochem), 2 μM XAV939 (Sigma-Aldrich), and 2 μM IWR-1 (Sigma-Aldrich), respectively, and incubated for 20 min at room temperature. Samples were assayed for proteasome activity with the Proteasome-Glo Chymotrypsin-like Cell-Based Assay (Promega) in a Spectramax M2 reader (Molecular Devices). To assess the effect of ADP-ribosylation on 20S and the 26S proteasome activities, we programmed purified bovine 20S and 26S

(D) DmPI31 modification promotes de novo assembly of the 26S proteasome. Purified bovine 19S and 20S proteasome particles (1 μg each) were incubated with His-DmPI31, His-DmPI31$^{\Delta HbYX}$, and His-DmPI31^{ADPR} (5 μg each) in the presence of ATP, followed by native gel analysis for the assessment of the effect of modified DmPI31 on proteasome assembly.

(E) Modified DmPI31 rescues XAV939-induced defects in 26S proteasome assembly to a greater extent than its unmodified control. 200 μg of embryo extracts treated with the TNKS-inhibitor were supplemented with 5 μg of respective proteins followed by native gel analysis.

In (D) and (E), native gels were analyzed via western blot with anti-Rpt3 (for 26S proteasome) and anti-α7 (for 20S proteasome). Chemoluminescence was quantitated with ImageQuant LAS 4000 (GE Healthcare). 26S proteasome assembly status is reported as the relative intensity of Rpt3 bands.

(F) A model for proteasome regulation by ADP-ribosylation. PI31 is a conserved proteasome-regulatory protein that is required for normal 26S proteasome activity in vivo and organismal viability (Bader et al., 2011). ADP-ribosylation of PI31 by TNKS, which transfers the ADP-ribose group from NAD^+, activates PI31 and leads to increased proteolytic activity of the 26S proteasome by promoting its assembly. Specifically, ADP-ribosylation of PI31 causes decreased affinity for 20S proteasome α subunits and dislodges PI31 from 20S particles. On the other hand, ADP-ribosylation increases binding of PI31 to the assembly chaperones dp27 and dS5b and, thereby, sequesters them away from 19S regulatory particles. Altogether, this promotes 26S proteasome assembly and facilitates the breakdown of intracellular proteins. Inhibition of TNKS by small-molecule compounds, such as XAV939, blocks this process and reduces 26S proteasome activity. Therefore, TNKS inhibitors represent a distinct class of proteasome inhibitors that target the assembly of 26S proteasomes.

Also see Figure S6.

proteasomes (0.1 μg; UBPBio) in PIPES buffer + 2 mM ATP were programmed with 2 μg of His-DmPI31^{WT}, His-DmPI31$^{\Delta HbYX}$, and His-DmPI31^{ADPR}, and incubated for 20 min at room temperature. Samples were assayed for proteasome activity as described above.

Gel Filtration Assay

For gel filtration assays, 2 mg of 0–2 hr WT embryo extracts prepared with the PIPES buffer + 2 mM ATP were centrifuged twice at 14,000 rpm for 20 min at 4°C prior to being treated with 50 μg His-DmPI31, 50 μg His-DmPI31$^{\Delta HbYX}$, 50 μg His-DmPI31$^{ADP\text{-}ribosylation}$, 1% DMSO, 5 μM MG132, 2 μM XAV939, and 2 μM Olaparib (LC Laboratories) for 30 min. Subsequently, gel filtration assay was performed using Superose 6 10/300GL FPLC column (GE Healthcare). We collected 500 μl fractions using a 0.250 ml/min flow rate. Purified bovine 19S, 20S, and 26S proteasomes (UBPBio), thyroglobulin, and BSA were used as standards.

In Vitro ADP-Ribosylation Assay and Purification of His-DmPI31^{ADPR}

Recombinant His-DmPI31 (1 μg), FLAG-dTNKS (0.1 μg), and His-dTNKS$^{\Delta PARP}$ (0.1 μg), along with biotin-NAD$^+$ (3.5 μM; Trevigen) were incubated for 5 hr at 37°C and subjected to SDS-PAGE followed by western blotting with Streptavidin HRP (Thermo Scientific; 1:10,000). The effects of XAV939 on dTNKS and dPARP were assessed by incubating biotin-NAD$^+$ and FLAG-dTNKS or FLAG-dPARP mixtures with either 0.1% DMSO or 0.1 μM XAV939. For the purification of His-DmPI31^{ADPR} protein, in vitro ADP-ribosylation of recombinant His-DmPI31 protein was performed as above. This was followed by serial purifications with the use of TALON Metal Affinity Resin (Clontech Laboratories) and SoftLink Soft Release Avidin Resin (Promega) per the manufactures' instructions.

Native Gel Analysis

To analyze proteasome assembly status, we resolved 200 μg of embryo extracts exposed to different experimental conditions + 2 mM ATP using 5% Tris-HCl acrylamide gels (Bio-Rad) under nondenaturing conditions and transferred the extracts onto a 0.22 μm PVDF membrane. Western blot analyses with the specific antibodies were performed as described above. Concentrations of various reagents were 1% DMSO, 5 μM MG132, 2 μM XAV939, purified bovine 19S and 20S (1 μg), and 5 μg of His-DmPI31^{WT}, His-DmPI31$^{\Delta HbYX}$, and His-DmPI31^{ADPR} proteins.

Fly Strains

UAS-DTS5 and UAS-DTS7 lines were obtained from J. Belote, dTNKS (21930, 21932, and 106238), dp27 (28527, 47762, and 47763), dS5b (104492), and dRpn14 (32697 and 32698) RNAi lines were obtained from the Vienna *Drosophila* RNAi Center and the Bloomington Drosophila Stock Center.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.03.040.

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