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A conserved F-box–regulatory complex controls proteasome activity in *Drosophila*

Maya Bader[1], **Sigi Benjamin**[1], **Orly L. Wapinski**[1], **David M. Smith**[2], **Alfred L. Goldberg**[2], and **Hermann Steller**[1]

[1] Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, NewYork, NY 10021, USA

[2] Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA

Summary

The Ubiquitin-Proteasome System catalyzes the degradation of intracellular proteins. Although ubiquitination of proteins determines their stabilities, there is growing evidence that proteasome function is also regulated. We report the functional characterization of a conserved proteasomal regulatory complex. We identified DmPI31 as a binding partner of the F-box protein Nutcracker, a component of an SCF ubiquitin ligase (E3) required for caspase activation during sperm differentiation in *Drosophila*. DmPI31 binds Nutcracker via a conserved mechanism that is also used by mammalian FBXO7 and PI31. Nutcracker promotes DmPI31 stability, which is necessary for caspase activation, proteasome function and sperm differentiation. DmPI31 can activate 26S proteasomes *in vitro*, and increasing DmPI31 levels suppresses defects caused by diminished proteasome activity *in vivo*. Furthermore, loss of DmPI31 function causes lethality, cell-cycle abnormalities and defects in protein degradation, demonstrating that DmPI31 is physiologically required for normal proteasome activity.

Introduction

The Ubiquitin-Proteasome System (UPS) is the major system for selective degradation of proteins in eukaryotic cells, and is implicated in the regulation of most cellular processes, including cell-cycle control, transcription, signal transduction, inflammation and cell viability (Finley, 2009; Glickman and Ciechanover, 2002). Protein substrates are targeted for degradation by a set of enzymes that attach multiple molecules of the small protein ubiquitin (Ub). These include the Ub-activating enzymes (E1s), Ub-conjugating enzymes (E2s), and a variety of Ub-protein ligases (E3s), which are responsible for substrate recognition and specificity (Glickman and Ciechanover, 2002).

The 26S proteasome is a large protease complex composed of two major components: the core or catalytic particle (CP or 20S), a cylindrical particle within which polypeptides are hydrolyzed to small peptides. The 20S proteasome is associated with one or two regulatory particles (RP or 19S) that bind the ubiquitinated substrate, unfold it and translocate the

Corresponding author: Hermann Steller steller@rockefeller.edu, +1-212-327-7075.

extended polypeptide through the narrow entry channel of the CP (Coux et al., 1996; Demartino and Gillette, 2007; Finley, 2009). Recent studies have identified many loosely associated proteins of this structure, which may function as regulators or co-factors, but the precise activities of most of these proteins remains elusive (Besche et al., 2009; Finley, 2009).

An added level of proteasomal regulation is the replacement of the 19S RP with alternative activator complexes, such as REGαβ (PA28αβ or 11S), REGδ (PA28δ) and Blm10 (PA200) (Ma et al., 1992; Masson et al., 2001; Tanahashi et al., 1997; Ustrell et al., 2002). These complexes can bind to either or both end of the 20S particle, and enhance the entry and degradation of small peptide substrates (Stadtmueller and Hill; Whitby et al., 2000). Given the variety and complexity of these regulatory components, and the growing number of proteasome associated proteins being identified, it is clear that proteasomes are much more complicated, more diverse and highly regulated structures than previously thought (Demartino and Gillette, 2007).

Here we describe the identification and functional characterization of a novel proteasome regulatory complex that controls caspase activation and spermatogenesis in *Drosophila*. Caspases are the key executioners of apoptosis, but during terminal sperm differentiation they are utilized for an apoptosis-like process that eliminates excess organelles and cytoplasm (Arama et al., 2003). Non-lethal caspase activity is also used in other examples of cellular remodeling, such as neuronal pruning (Kuo et al., 2006; Nikolaev et al., 2009; Williams et al., 2006). Neuronal remodeling is important for normal development, but has also been implicated in neurodegenerative diseases (Nikolaev et al., 2009; Raff et al., 2002; Tai and Schuman, 2008). Therefore, insights into cellular remodeling and the proteases that mediate it will likely contribute to a better understanding of these diseases.

From a screen for genes that control caspase activation during terminal sperm differentiation, we isolated the F-box protein Nutcracker, the substrate-binding unit of an SCF Ub-ligase complex (Bader et al., 2010). Mutations in *nutcracker* abrogate caspase activity and prevent the progression of cellular remodeling, resulting in sterility. Here we describe the identification and characterization of a Nutcracker-binding partner, DmPI31, discovered in a proteomic screen for Nutcracker interactors. We show that DmPI31 stability is promoted by Nutcracker, and that they functionally cooperate to activate caspases and drive sperm differentiation. Furthermore, we find that DmPI31, which was originally described as a proteasome inhibitor in mammalian systems, can activate purified 26S proteasomes *in vitro* (Chu-Ping et al., 1992; McCutchen-Maloney et al., 2000; Zaiss et al., 1999; Zaiss et al., 2002). Elevated levels of DmP31 can suppress phenotypes caused by reduced proteasome activity *in vivo.* Finally, loss-of-function mutations in DmPI31 are lethal, demonstrating that this protein has an essential physiological function. DmPI31 mutants accumulate poly-ubiquitinated proteins and display cell-cycle abnormalities, suggesting that DmPI31 is physiologically required for normal proteasome activity *in vivo*. These findings reveal a conserved mode of proteasome regulation.

Results

Proteomic screen to identify Nutcracker interacting proteins

Nutcracker is a *Drosophila* F-box protein previously identified in a screen for mutants that fail to activate caspases during spermatid differentiation (Bader et al., 2010). To further understand its role in caspase activation and spermatogenesis, we sought to identify interacting partners by co-immunoprecipitation (co-IP) of Nutcracker followed by identification of associated proteins using mass-spectrometry (Figure 1A).

A protein-A(PrA) tagged version of full-length Nutcracker (PrA-ntc) was expressed in both *Drosophila* testes and S2 cells. After co-IP, the interacting proteins were identified using MS/MS mass-spectrometry (Figures 1B and S1A). This revealed a group of proteins belonging to the UPS, primarily proteasome subunits (alpha1 and alpha7). Co-IP experiments confirmed that Nutcracker can associate with proteasomes *in vivo* (Figure 1C). In addition, we identified a putative proteasome inhibitor, DmPI31 (CG8979), as a binding partner of PrA-ntc in both S2 cells and testes (Figure 1B&D and S1A). The interaction between Nutcracker and DmPI31 appears to be direct since both proteins can bind each other in a yeast two-hybrid assay (Giot et al., 2003). Next, we generated an antibody against recombinant DmPI31 and confirmed that this interaction occurs *in vivo* (Figure 1D). We chose to focus on DmPI31 because of its potential role in regulating the proteasome.

DmPI31-Nutcracker interaction depends on the conserved FP domain

We previously showed that the F-box domain of Nutcracker is important for binding Cullin1 and SkpA (Bader et al., 2010). To determine if the F-box domain is also required for Nutcracker-DmPI31 binding, we performed co-IP experiments using testes expressing either the original PrA-ntc, or a truncated version that lacks the F-box domain (PrA-ntcΔF). Co-IP of both PrA-ntc and PrA-ntcΔF resulted in enrichment of DmPI31 (Figure 1D). This suggests that the interaction between Nutcracker and DmPI31 is independent of the F-box domain.

To further investigate the nature of Nutcracker-DmPI31 interaction, we looked at a related, structurally defined association between the mammalian F-box protein FBXO7 and PI31 (Kirk et al., 2008). Nutcracker has some homology with FBXO7 and they share a critical conserved valine (Figure S1B) that is required for FBXO7-PI31 binding. We mutated this valine in Nutcracker (V>E) to examine its significance in the Nutcracker-DmPI31 interaction. As shown in Figure 1E, significantly less endogenous DmPI31 was bound to the mutant Nutcracker protein than the wild type form. These results show that the conserved FP domain is essential for interaction with DmPI31.

DmPI31 is highly expressed in the testes and is localized to Individualization Complexes

DmPI31 is the *Drosophila* homologue of mammalian PI31 proteins, which have been found to inhibit the activity of purified 20S proteasomes (Chu-Ping et al., 1992; McCutchen-Maloney et al., 2000; Zaiss et al., 1999). The *Drosophila* homologue shares over 45% homology with these proteins (Figure 2A). DmPI31 mRNA is expressed throughout the *Drosophila* lifecycle, but drops substantially in the adult female (Arbeitman et al., 2002; Gauhar, 2008). Semi-quantitative RT-PCR experiments demonstrated that mRNA levels in adult females are equivalent to those of *son-of-oskar* males, which lack germ cells and functioning testes. This suggests that the majority of adult mRNA resides in testes (Figure 2B).

In order to determine the expression and intracellular localization of DmPI31, testes were stained with the DmPI31 antibody. At the onset of terminal sperm differentiation, in a process called “individualization”, actin-rich cones form around the nuclei to create the Individualization Complex (IC) (Figure 2C and 2D) (Fuller, 1993). As the IC moves, most of the cytoplasm and excess organelles are expelled in an inflated structure called the cystic bulge (CB). Nutcracker protein is highly localized around the IC’s actin cones in the CB (Figure 2E and (Bader et al., 2010)). DmPI31 is distributed in a very similar pattern (Figure 2E and 2F), and both proteins can co-localize upon expression in cultured cells (Figure S2). This suggests that DmPI31 and Nutcracker localize to the same cellular compartment during individualization, consistent with their physical interaction *in vivo*.

We also generated a mCherry-DmPI31 fusion protein driven by the *dmPI31* endogenous promoter. This reporter displayed a similar subcellular distribution as the antibody staining, indicating that the fusion protein is properly localized (Figure 2G). Furthermore, mCherrry-DmPI31 was able to rescue the lethality of dmPI31 mutants (see below). Therefore, this fusion protein can substitute for endogenous DmPI31 protein and appears to be a faithful reporter for DmPI31 localization and behavior.

Nutcracker controls DmPI31 protein levels

Since the Nutcracker-DmPI31 interaction is independent of the F-box domain, we asked whether DmPI31 might be a substrate of the Nutcracker-containing SCF ubiquitin ligase. If DmPI31 is a substrate for Nutcracker-mediated degradation, we would have expected DmPI31 to accumulate in the mutants. In contrast, DmPI31 levels were greatly reduced in testes of both Nutcracker-null (nutcracker^{07259}) or truncated F-box (*nutcracker*^{ms771}) mutants (Figure 3A) (Bader et al., 2010). Instead, smaller molecular weight products were detected. The full-length DmPI31 band was fully restored in *nutcracker*$^{ms771\,-/-}$ flies that express wild type Nutcracker (*nutcracker* "rescue" transgenes: hsp83-nutcracker). This indicates that DmPI31 is cleaved in a *nutcracker* mutant background, suggesting that Nutcracker positively regulates DmPI31 protein stability. Also, since a F-box-truncated form of Nutcracker (*nutcracker*$^{ms771\,-/-}$) still destabilizes DmPI31, it appears that the F-box domain is important for stabilizing DmPI31. Finally, we investigated whether the cleavage of DmPI31 in a *nutcracker* mutant background depends on caspases or proteasome activity and found no evidence for an involvement of these proteases (Figure S3A).

The amino-terminal region of PI31 is structured and contains the FP domain, while the carboxy-terminal end, which contains proline-rich sequences, is unstructured (Kirk et al., 2008; McCutchen-Maloney et al., 2000) (Figure 2A). We therefore investigated whether the cleaved form of DmPI31 seen in lysates of *nutcracker* mutants is the result of random proteolysis, or cleavage of a specific portion of the protein. For this purpose, we expressed mCherry-DmPI31 in a *nutcracker*$^{771\,-/-}$ mutant background and probed with an anti-mCherry antibody. If cleavage of DmPI31 were near the C-terminus, the resulting band would decrease by approximately 10kD. On the other hand, if the cleavage were near the N-terminus, we would expect the molecular weight to decrease by 20KD (see diagram in Figure 3B). The size of mCherry-DmPI31 in a *nutcracker* mutant background decreased by 10KD. Since mCherry-DmPI31 is biologically active, we assume that the cleavage site is not affected by the presence of the mCherry tag and conclude that the C-terminus of DmPI31 is truncated in a *nutcracker* mutant background. The C-terminus of mammalian PI31 is required for proteasome interaction, so loss of *nutcracker* function is predicted to inactivate DmPI31 (McCutchen-Maloney et al., 2000).

Next we investigated if the interaction between DmPI31 and Nutcracker is lost upon truncation of the DmPI31 C-terminal domain. We repeated the co-IP experiment, but this time we expressed PrA-ntc in the testes of the *nutcracker*$^{ms771\,-/-}$ mutant flies (Figure 3C). PrA-ntc was able to IP both the full length and cleaved forms of DmPI31, indicating that the cleaved form is folded and capable of interacting with Nutcracker. This suggests that the phenotypes observed in *nutcracker* mutants result from the cleavage of DmPI31, and not simply from the inability of both proteins to interact.

Nutcracker and DmPI31 act together to activate caspases and control sperm differentiation

We asked if the interaction between the two proteins is important for their biological activity *in vivo*. For this purpose, we mutated the FP domain of Nutcracker and tested whether a transgene carrying this mutation can rescue *nutcracker*$^{ms771\,-/-}$. Although both constructs were expressed at similar levels (Figure S3B), hsp83-nutcrackerV-E transgenic flies were

unable to rescue the *nutcracker*$^{ms771\,-/-}$ sterility phenotype (Table 1). When we stained testes from these males with the caspase3 antibody, we noticed that some staining was restored, demonstrating that the mutated construct was partially functional (data not shown). However, it appears that full Nutcracker activity requires binding to DmPI31.

We then investigated if the inability of the mutated rescue construct to restore fertility is caused by persistent DmPI31 cleavage. We found that hsp83-nutcrackerV-E;*nutcracker*$^{ms771\,-/-}$ still displayed DmPI31 cleavage, albeit less than in the original *nutcracker*$^{ms771\,-/-}$ homozygote mutant line (Figure 3D). This indicates that the interaction between Nutcracker and DmPI31 is important for Nutcracker's role in stabilizing DmPI31, and that this stabilization is crucial for proper sperm differentiation.

If Nutcracker functions through DmPI31 stabilization to promote caspase activation and sperm differentiation, it may be possible to at least partially compensate for the loss of Nutcracker function by expressing DmPI31. We over-expressed DmPI31 in a *nutcracker*$^{ms771\,-/-}$ mutant background and observed partial restoration of caspase staining (Figure 3E). This suggests that Nutcracker activates caspases, at least in part, by stabilizing DmPI31. According to this interpretation, Nutcracker is dispensable for caspase activation if sufficient amounts of full-length DmPI31 are expressed in spermatids. Taken together, our results demonstrate that Nutcracker and DmPI31 need to physically interact for their full biological activities.

DmPI31 can stimulate 26S proteasomes *in vitro*

PI31 proteins were identified as inhibitors of the catalytic particle of the proteasome based on the ability of recombinant bovine PI31 to inhibit purified 20S proteasomes (Chu-Ping et al., 1992; McCutchen-Maloney et al., 2000; Zaiss et al., 1999). Subsequent studies also demonstrated PI31's ability to compete with 19S and 11S regulatory particles which resulted in a net inhibitory effect (McCutchen-Maloney et al., 2000). In order to understand the molecular role of *Drosophila* PI31 in regulating spermatogenesis, we investigated its effect on the proteasome's peptidase activity. Recombinant DmPI31 was purified from *E. coli* and added to purified mammalian 20S proteasomes. As expected, DmPI31 inhibited 20S activity (Figure 4A). Surprisingly, when purified DmPI31 was added to mammalian 26S proteasomes, its peptidase activity was enhanced up to 3-fold (Figure 4A). Thus, when the 20S CP is associated with the 19S RP (and possibly various other interacting proteins), DmPI31 enhances proteasome function, in contrast to the inhibitory effect on purified 20S CP.

The capacity of 26S and 20S proteasomes to degrade small peptides is determined by whether the gate for its substrate-entry channel is in its open conformation, and in the 26S particle, this entry is regulated by the ATPases in the 19S (Smith et al., 2005). Such an effect on gating could be mediated by DmPI31's C-terminus Hydrophobic-Tyrosin-X (HbYX) motif that in 19S ATPases allows interaction with the proteasome and induces gate opening (Rabl et al., 2008; Smith et al., 2007). Consistently, we found that mutant DmPI31 protein lacking the HbYX motif could still activate the 26S, but with a reduced efficiency (Figure 4B). This suggests that while the HbYX domain is important for proteasome activation, it may not be the only domain that mediates activation and binding. The difference in DmPI31 effect on the 20S vs. 26S suggests that other domains in DmPI31 may bind to the 19S regulatory subunit as well. Collectively, the *in vitro* data indicate that DmPI31, despite its original designation as a proteasome inhibitor, can function as an activator of the 26S proteasome, which is the dominant form *in vivo* (Besche et al., 2009).

Ectopic expression of DmPI31 increases proteasome activity *in vivo*

We conducted a series of genetic studies to determine whether DmPI31 can affect proteasome activity *in vivo.* First we analyzed whether expression of DmPI31 had an effect on phenotypes caused by impaired proteasome function. Targeted expression of the dominant temperature sensitive proteasome alleles UAS-DTS5 and UAS-DTS7 with GMR-Gal4 at 29°C causes a small, rough eye phenotype (Belote and Fortier, 2002) (Figure 5B and 5C). Co-expression of DmPI31 with these constructs suppressed this phenotype (Figure 5D). This appears to be a direct effect on boosting proteasome activity because expression of DmPI31 did not increase the expression of proteasome subunits (Figure 5E). Conversely, reducing *DmPI31* function significantly enhanced the phenotype of the DTS alleles (Figure 5F–5J). Whereas expression of either DTS allele at 25°C caused no detectable eye phenotype, down-regulation of DmPI31 via RNAi caused a strong eye phenotype in the background of both DTS alleles (Figure 5F–5J). At 29°C, this allelic combination results in organismal lethality (Figure 5K–5N). These results show that DmPI31 can modify proteasome activity *in vivo* and suggests that DmPI31 functions as a proteasome activator.

DmPI31 is physiologically required for proteasome function

To determine the physiological function of DmPI31, we generated loss-of-function mutations in this gene by homologous recombination (Gong and Golic, 2003). *dmPI31* mutants are recessively lethal and die at the late third instar larval/early pupal transition, a developmental stage where many cell cycle mutants are lethal (Gatti and Baker, 1989; Orr-Weaver, 1994). Mutant larvae also developed melanotic tumors, were slower to develop and smaller. All these phenotypes were rescued by transgenic expression of dmPI31 (using UASt-HA-PI31 transgenic flies under the control of the tubulin-Gal4, tubulin Gal80^{ts} promoters at 23°C), demonstrating that the mutant phenotypes are indeed caused by loss of *dmPI31* function. On the other hand, high-level over-expression of DmPI31 caused lethality (Table S1). This indicates that cells are highly sensitive to the amounts of PI31, since both elimination of the gene and over-expression are detrimental and cause lethality. We also generated transgenic fly lines to ectopically express various DmPI31 mutants for structure/function studies (Table S1). Consistent with our previous results, truncation of the C-terminal region of DmPI31 abrogated the ability of DmPI31 to stimulate proteasome activity.

Amongst the many cellular processes regulated by the UPS is cell cycle progression. We therefore examined whether *dmPI31* mutants have cell cycle defects, focusing on the male germ line. By expressing DmPI31 transgenically in somatic cells we were able to rescue organismal lethality and generate viable “mosaic” adults that lack dmPI31 function in germ cells because germ cells use a different *tubulin* promoter than somatic cells (Hoyle et al., 1995; Kemphues et al., 1980). When the testes of these flies were dissected they appeared irregular and exhibited a meiotic arrest phenotype, characterized by the abundance of underdeveloped 16 cell stage cysts, which fail to undergo proper meiosis (Lin et al., 1996). Antibody staining against DmPI31 confirmed the absence of protein in both the germ-line cells (GSCs) and primary spermatocytes (Figure 6A).

Next, we stained testes with cell cycle markers. BrdU staining at the tip of the testes closest to the hub was similar to the wild-type, indicating that mitosis of germ-line cells proceeded normally (Figure 6B). In contrast, when we stained with the phospho-histone3 (PH3) antibody, which labels dividing nuclei, we did not detect cysts in the 32-cell stage, suggesting that these cells are not undergoing meiosis (Figure 6C). Furthermore, staining with the Vasa antibody, which is specific for germ cell progenitors, showed that loss of DmPI31 function prevents germ cell differentiation and leads to persistence of progenitor identity (Jin et al., 2005) (Figure 6D). Moreover, we saw that CyclinB, a protein important

for both mitotic and meiotic cell cycles, that is degraded by the proteasome during the final stages of meiosis, also persisted in these cells (Fuller, 1998) (Figure 6E and S6). These results are consistent with the idea that loss of DmPI31 impairs proteasome function and that DmPI31 is a proteasome activator.

To further test whether *dmPI31* mutants have reduced proteasome activity, we stained testes with the FK2 antibody, which detects Ub-conjugated proteins and can therefore be used to visualize poly-ubiquitinated, non-degraded proteins that accumulate when 26S proteasome function is impaired (Fujimuro et al., 1997; Haas et al., 2007). In wild type cells, FK2 staining was diffuse in the cytoplasm and strong in the nucleus of meiotic cells (Figure 6F). In contrast, in *dmPI31* mutant cells, punctate staining was seen in most cells, and in some cases in one or two nuclei of each cyst. This pattern of staining suggests that proteasome activity is defective, and that Ub-conjugates accumulate as intracellular aggregates. Taken together, our results show that DmPI31 is required for proper protein degradation and cell cycle progression, supporting a role of this protein as a proteasome activator.

Discussion

A conserved proteasome regulatory complex is required for cellular remodeling

We identified and characterized a proteasome regulatory complex that combines two distinct components of the UPS – an F-box protein, which usually catalyzes ubiquitin-conjugation to a set of substrates, and a proteasome regulator, which promotes substrate hydrolysis by the proteasome. Although several studies have reported the physical association between certain Ub-ligases and the 26S proteasome, we now demonstrate a clear functional significance of this interaction *in vivo* (Demartino and Gillette, 2007; Finley, 2009). In particular, we show that the F-box protein Nutcracker and the proteasome regulator DmPI31 function together as a complex to stimulate proteasome activity during normal development. This elevated proteasome activity is required for non-apoptotic caspase activation and sperm differentiation in *Drosophila*. The interaction of DmPI31 and Nutcracker is mediated through their FP domain, a conserved domain found in mammalian homologues (Kirk et al., 2008). Therefore, it is possible that a similar mode of proteasomal regulation operates in mammals.

Nutcracker controls caspase activation and sperm individualization by promoting DmPI31 stability

We initially considered that DmPI31 is a substrate of the Nutcracker-containing SCF E3-ligase complex. However, DmPI31 protein does not accumulate in *nutcracker* mutants, as would be expected if it was a degradation substrate. Instead, a *nutcracker* mutant that carries a stable but truncated F-box domain results in the cleavage and truncation of DmPI31. Hence, the Nutcracker F-box domain is required for the stability of full-length DmPI31 and the proteasome-activating activity of this protein (Figure 7). Furthermore, stabilization of DmPI31 by Nutcracker is required for normal caspase activation, sperm differentiation and male fertility. First, loss of Nutcracker function causes defects in caspase activation and male sterility (Bader et al., 2010). Second, a *nutcracker* transgene with reduced ability to bind DmPI31 failed to protect DmPI31 from cleavage and did not rescue *nutcracker* mutants. This suggests that binding of Nutcracker to DmPI31 is critically important to positively regulate DmPI31 by stabilizing this protein. Additionally, over-expression of DmPI31 in a *nutcracker* mutant background was sufficient for caspase activation (Fig. 3E). This indicates that physical interaction with DmPI31 is important for Nutcracker activity, and that Nutcracker acts upstream of DmPI31 to control its stability and function. Since general over-expression of DmPI31 is lethal, it appears that the levels of active (full-length) DmPI31 need to be carefully regulated.

DmPI31 is a proteasome activator *in vivo*

Contrary to the original identification of PI31 as a proteasome inhibitor (PI), the physiological function of DmPI31 is to stimulate proteasome activity *in vivo.* This stimulatory effect of PI31 on proteasomes appears to be direct because DmPI31 can also activate purified 26S proteasomes *in vitro.* The ability of DmPI31 to enhance the degradation of standard tetrapeptide substrates implies a role in promoting the opening of the substrate-entry channel, a property exhibited by other proteasome activating complexes (Rabl et al., 2008; Smith et al., 2007). In support of this idea, DmPI31's C-terminal HbYX motif, which resembles the proteasome-interacting, gate-opening domains on the proteasome-regulatory ATPases, is also important for DmPI31 activity (Rabl et al., 2008; Smith et al., 2007).

The physiological role of DmPI31 as an essential proteasome activator is supported by the phenotypes of *dmPI31* loss-of-function mutants. Loss of *dmPI31* function causes lethality, indicating that this gene has a vital function. Furthermore, germ cells lacking *dmPI31* fail to undergo meiosis and maintain stem cell identity. These phenotypes are consistent with defects in protein degradation (Alessandrini et al., 1997; Fuller, 1998). Accordingly, we observed that poly-ubiquitinated proteins accumulate in *dmPI31* mutant germ-cells, proving strong evidence that DmPI31 is required for normal proteasome activity *in vivo*. Finally, a modest elevation of DmPI31 protein was able to suppress phenotypes caused by reduced proteasome activity, indicating that DmPI31 can boost proteasome activity *in vivo*. Taken together, all these results argue that DmPI31 plays an important physiological role as a proteasome activator.

Our findings may have important general implications for the role of regulated proteolysis during cellular remodeling. Sperm differentiation involves a major reduction in cell volume and can be viewed, from a cell biological standpoint, as 'programmed cell atrophy' (Arama et al., 2006; Bader et al., 2010; Fabrizio et al., 1998; Noguchi and Miller, 2003; Zhong and Belote, 2007). Similar dramatic morphological changes and organelle breakdown also occur during the differentiation of other cell types, for example during neuronal pruning (Lecker et al., 2006; Ventadour and Attaix, 2006). Consequently, it is possible that proteasomes are positively regulated in these cases as well. Furthermore, intracellular proteolysis mediated by both proteasomes and caspases is associated with various pathologies that involve cell and tissue wasting, including neural degeneration and myopathies (Lecker et al., 2006; Raff et al., 2002; Saxena and Caroni, 2007). Therefore, insights into the regulation of protein degradation are highly relevant for a wide range of human diseases. In the future, it will be in interesting to investigate if a mechanism similar to what we describe here controls proteasome activity in mammals, and whether it contributes to human diseases that are associated with excessive protein turnover.

Experimental Procedures

Fly strains

yw flies were used as wild-type controls. The Zuker mutant Z3-4692 (ms771) was obtained from C.S. Zuker; the *son-of-oskar* osk[301]/TM3 and osk[CE4]/TM3 lines from R. Lehmann; w1118;70FLP,70ISceI and w1118;70FLP lines from L.B. Vosshall; tubulin-Gal4 from HD. Ryoo; UAS-DTS5 and UAS-DTS7 were obtained from J. Belote; the deficiency lines Df(2R)BSC199 and Df(2R)ED2247 and the PBac insertion PBac{WH}CG10855^{f07259} from the Bloomington Stock Center, as were tubulin-Gal80^{ts} and actin-Gal4. DmPI31 RNAi lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC).

Immunoprecipitations

Testes-IP was conducted as in (Bader et al., 2010). For S2 IP, cells were co-transfected with Actin-Gal4 and different UAS constructs (Fugene6, Roche) and left for 36–48 hrs. Cells were washed twice in ice-cold PBS, lysed in 200μl ice-cold 1% NP-40 in PBS, left on ice for 15 minutes, then spun at 14,000 rpm for 15 minutes at 4°C. The IP was then conducted similarly to the testes IP, except the washes were done with ice-cold PBS. 0.5–1mg of total-protein was used for IP-Western, and 7–10mg for mass-spectrometry identification.

Mass spectrometry analysis

After co-IP, proteins were resolved by SDS-PAGE (4%–12% gradient) and stained with Coomassie Blue (GelCode Blue; Pierce). Visible bands were excised and subjected to trypsin digestion. The resulting peptides were extracted, and proteins were identified by mass spectrometry at the Rockefeller University Proteomics Resource Center. Peptides sequences were analyzed using the MASCOT search engine (http://www.matrixscience.com/).

Western Blot Analysis

Western blot analysis was conducted as in (Bader et al., 2010) with anti-DmPI31[sera], 1:1000, anti-HA, 1:5000 (Roche), anti-alpha7, 1:200 (Biomol), mCherry 1:1000 (clontech), CyclinB (1:1000) Santa Cruz.

Antibody generation and tissue staining

Cleaved effector caspase antibody staining of testes was as in (Bader et al., 2010). Anti-DmPI31 was created in guinea-pigs with full-length recombinant DmPI31 (Cocalico Biologicals, Inc.), and staining was as described in (Hime et al., 1996) with serum diluted 1:250. Staining of whole mount testes and BrdU incorporation followed (Beall et al., 2002), except CyclinB staining was as in (Baker and Fuller, 2007). The following antibodies and dilutions were used: mouse anti-BrdU-FITC 1:100 (BD-Pharmingen), rabbit anti-Vasa 1:100 (a gift from R. Lehmann), mouse anti-CyclinB clone F2F4 1:10 (DSHB) and mouse FK2 1:100 (Stressgen).

Proteasome activity assay

To measure proteasome activity, the fluorogenic peptide substrate Suc-LLVY-amc (Enzo Life Sciences, maintained in DMSO) was used at a final concentration of 100 μM in reaction buffer (50mM Tris, 5% glycerol). Hydrolysis of suc-LLVY-amc was monitored at λ_{ex} 380 nm and λ_{em} 440 nm. All reactions were conducted in a 96-well plate and read on a SpectraMax M2 micro-plate reader (Molecular Devices). For experiments on 20S proteasomes, 0.05–0.15μg of purified Bovine 20S was used per 100μl reaction, at 37°C. For experiments on 26S proteasomes, 0.05μg of purified Bovine 26S was used per 100 μl reaction, in the same buffer plus 10mM mgCl and 100μM ATP, at 37°C. K(apparent) was calculated with the standard ligand binding equation using sigmaplot (www.sigmaplot.com) with DmPI31 concentration at saturation as maximal affinity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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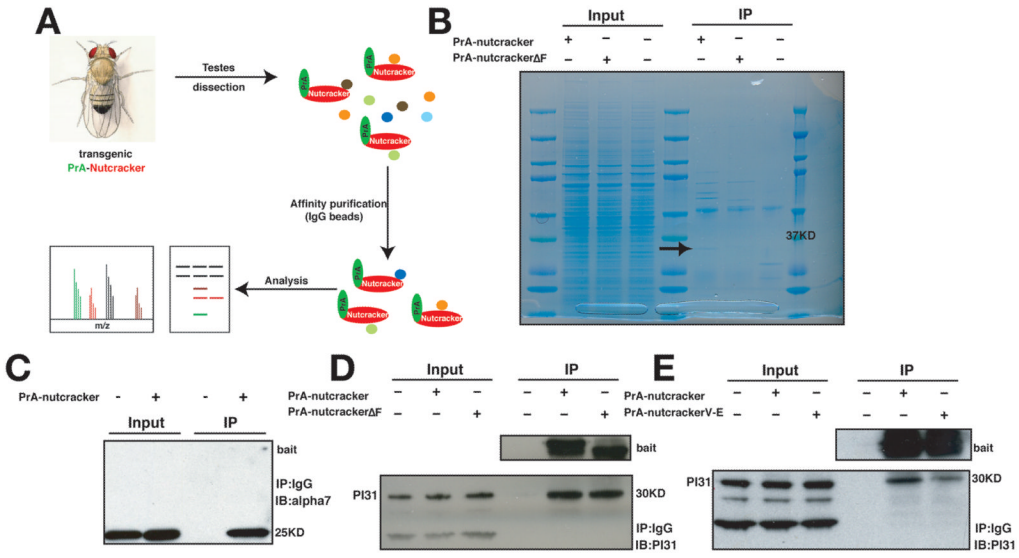

Figure 1. Proteomic screen for Nutcracker interacting proteins

[A] Scheme for testis-specific interactors of Nutcracker; PrA-nutcracker (PrA-ntc) was specifically expressed in testes with the Don-Juan (DJ) promoter. Testes were dissected, lysed, and incubated with IgG beads. Interacting complexes were eluted off the beads, and identified by either mass-spectrometry or Western-blot analysis. **[B]** SDS-PAGE commassie blots of Nutcracker interactors from cell lysate. PrA-ntc or PrA-ntc&DeltaF were expressed in S2 cells and used to make lysates for co-immunoprecipitation (co-IP) assays. Non-PrA expressing cells were used as a negative control. An arrow marks the DmPI31 band. See also Figure S1. **[C]** PrA-ntc is associated with proteasomes *in vivo.* PrA-ntc was expressed in testes and used for co-IP assays. Probing with an antibody against the proteasome subunit alpha7 shows that PrA-ntc forms a complex with proteasome proteins *in vivo*. **[D]** The Nutcracker-DmPI31 interaction is not dependent on the F-box domain. PrA-ntc or PrA-ntcΔF were expressed in testes and used to make lysates for co-IP assays. Non-PrA expressing wild-type testes (*yw*) were used as a negative control. DmPI31 binding was detected by Western-blot analysis using a DmPI31 antibody. **[E**] Nutcracker-DmPI31 binding is mediated by a specialized domain. A conserved valine in Nutcarcker that mediates the interaction between the human PI31 and the F-box protein FBXO7 was mutated to investigate its importance for DmPI31-Nutcracker binding. PrA-ntc or PrA-ntcV-E were expressed in S2 cells and lysates were used for co-IP. Non-PrA expressing cells were used as a negative control.

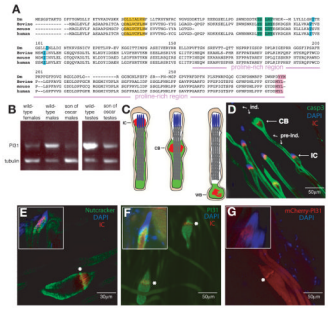

Figure 2. DmPI31 is localized to Individualization Complexes

[A] Multiple alignments of DmPI31 and three mammalian homologues (bovine, mouse, and human). DmPI31 shares overall 45% homology with the mammalian proteins, corresponding to 29% identity and 16% similarity. In particular, the DX_7H and YXLXY motifs, which are important for the PI31 structure, are highly conserved (labeled in yellow and green, respectively). Highlighted in blue are amino acids that mediate PI31-FBXO7 binding (Kirk et al., 2008). In pink is the Hydrophobic-Tyrosine-X (HbYX) motif. All accession numbers are listed in the M&M. **[B]** DmPI31 mRNA is abundant in adult testes. Semi-quantitative RT-PCR of dmPI31 mRNA transcripts taken from whole-body adult females, wild-type males or *son-of-oskar* males, which lack germ-cells. These are also compared to mRNA transcripts from wild-type testes or *son-of-oskar* testes. Beta-tubulin primers were used as control for total mRNA concentrations. Shown is cycle 25, 5 cycles before saturation. **[C]** Schematic diagram of spermatid individualization. An actin-based individualization complex (IC, red) forms around the elongated nuclei of 64 spermatids (gray) that are connected by cytoplasmic bridges. As the IC moves, cytoplasm (green) and organelles are collected in the cystic bulge (CB) and eventually discarded in the waste bag (WB), generating individual sperm devoid of most cytoplasm and organelles. **[D]** Caspase staining of individualizing cysts. Wild-type cysts were stained with DAPI (nuclei, blue), phalloidin (IC, red) and anti-active-caspase-3 (cytoplasm, green). **[E–G]** DmPI31 and Nutcracker localize to the same sub-cellular region during individualization. **[E]** Nutcracker staining during individualization as detected with an antibody that specifically recognizes this protein (Bader et al.). Nutcracker staining was seen at the base of the nuclei when the actin cones form around it (inset), and in a circular pattern around the cones in the CB (asterisks). **[F]** DmPI31 antibody staining. Like Nutcracker, DmPI31 protein is detected at the base of the elongated nuclei (inset), and co-localizes in a circular pattern with the actin cones (asterisks). Colocalization was also seen in cultured cells (Figure S2) **[G]** mCherry-DmPI31 fusion protein localization during individualization. This fusion protein is expressed under the control of *dmPI31* endogenous promoter and localized in a pattern virtually identical to DmPI31 antibody staining. mCherry-DmPI31 is seen in the combined cytoplasm of each cyst and at the base of fully elongated nuclei (inset), co-localizing with the actin cones as they move down the cyst (asterisks).

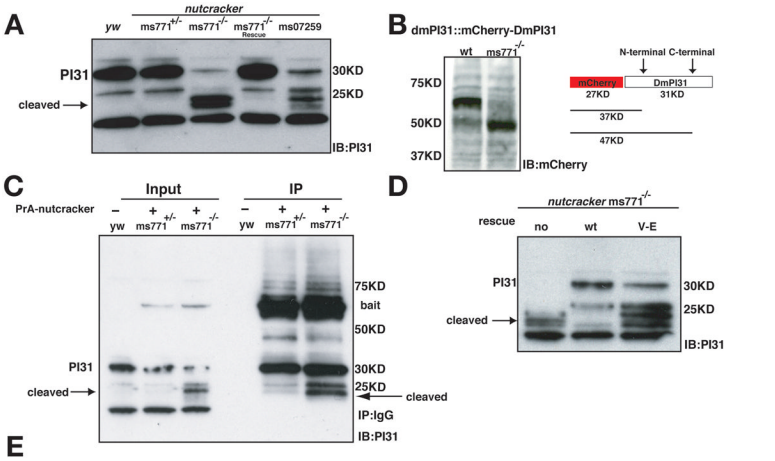

Table 1

Genotype	Fertility	Caspase3 staining	dmPI31 cleavage	Morphology
Wild-type (*yw*)	100%	yes	no	normal
ms771$^{(-/-)}$	0%	no	yes	no IC formed, failure to individualize
ms771$^{(-/-)}$ + nutcracker-WT	100%	yes	no	normal
ms771$^{(-/-)}$ + nutcracker-V-E	0%	yes	partial	incomplete individualization
ms771$^{(-/-)}$ + PI31	0%	yes		no IC formed failure to individualize

Figure 3. Nutcracker controls caspase activation and sperm individualization by promoting DmPI31 stability

[A] Mutations in *nutcracker* affect DmPI31 stability. Testes lysates of the indicated genotypes were used to detect steady-state DmPI31 protein levels. The cleaved form of DmPI31 found in *nutcracker* mutants is indicated by an arrow. The lowest molecular weight band is unspecific and serves as loading control. *yw* (wild type), ms771$^{+/-}$ (*nutcracker*^{ms771} heterozygote), ms771$^{-/-}$ (*nutcracker*^{ms771} homozygote), ms771$^{(-/-)}$ Rescue, (*nutcracker*^{ms771};hsp83-nutcracker), ms07259 (*nutcracker*^{07259} homozygote). **[B]** Mutations in *nutcracker* result in the C-terminal cleavage of DmPI31. Total lysates from either wild-type or *nutcracker*$^{ms771\,-/-}$ homozygote flies expressing mCherry-DmPI31 were used to detect the size of the fusion protein after cleavage. The diagram explains the expected molecular weights, which differ depending on whether the cleavage is N- or C-terminal. The observed cleavage fragment was ~50KD, and clearly not ~37KD. This indicates that the C-terminal domain of DmPI31 is truncated in *nutcracker* mutants. **[C]** The cleaved form of DmPI31 can physically associate with Nutcracker. PrA-ntc was expressed in testes of either *nutcracker*$^{ms771\,+/-}$ or *nutcracker*$^{ms771\,-/-}$ background and used to make lysates for co-IP. The cleaved form of DmPI31 found in *nutcracker* homozygote mutants is indicated by an arrow. Non-PrA expressing (*yw)* testes were used as a negative control. **[D]** DmPI31 stability depends on Nutcracker binding. Testes lysates from *nutcracker*$^{ms771\,-/-}$ homozygote mutants expressing the indicated rescue constructs were used to detect steady-state DmPI31 protein levels. hsp83-nutcracker and hsp83-nutcrackerV-E rescue constructs

are labeled wt and V-E respectively. The cleaved form of DmPI31 found in *nutcracker* mutants is indicated by an arrow. This cleavage is not dependent on caspase or proteasome protease activity (Figure S3A). The lowest molecular weight band is unspecific and serves as loading control. **[E]** Over-expression of DmPI31 is sufficient for caspase activation in the absence of *nutcracker* function. Active caspase-3 staining is shown in green and actin filaments (phalloidin staining) in red. Over-expression of DmPI31 in wild-type testes has no detectable effect on caspase-3 staining (left panel). Whereas *nutcracker*$^{ms771-/-}$ mutant testes lack caspase-3 staining (middle panel), expression of DmPI31 restores readily detectable caspase activity (right panel). **[**Table 1] Summary of *nutcracker* mutant rescue experiments. *nutcracker*$^{ms771-/-}$ (ms771$^{-/}$) phenotypes rescued by ectopic expression of either wild-type *nutcracker* (nutcracker-WT), a *nutcracker* construct containing a mutation that prevents binding to DmPI31 (nutcrackerV-E), or a DmPI31 construct (PI31). A control for equal expression is shown in Figure S3B.

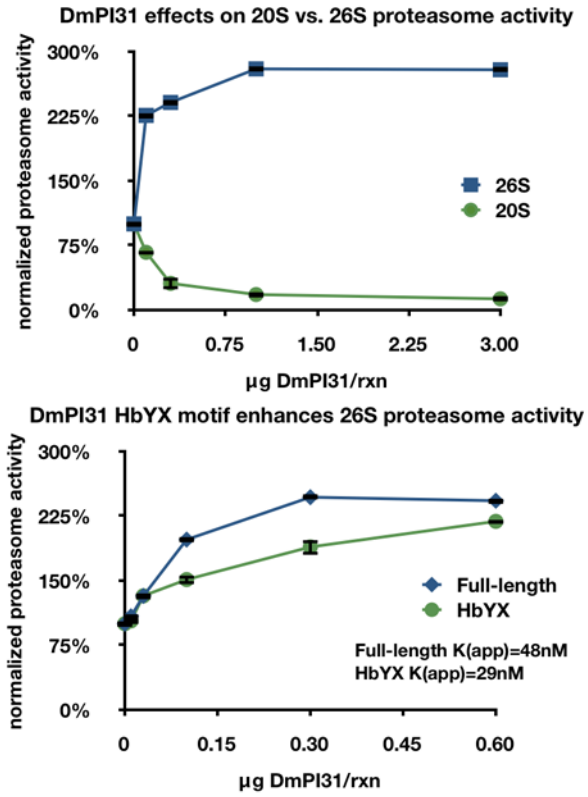

Figure 4. DmPI31 can stimulate proteasome activity *in vitro*

[A–B] *In vitro* proteasome activity assays using Suc-LLVY-AMC substrate. Rates are plotted relative to that of the control sample lacking DmPI31. Each experiment was repeated several times with similar results. Error bars represent the standard deviation from three independent readings. **[A]** DmPI31 is an inhibitor of 20S but an activator of the 26S proteasomes. Increasing concentrations of purified DmPI31 were used and the effect on the activity of purified bovine 20S or 26S proteasomes was monitored by rate of fluorogenic substrate hydrolysis. 0.15µg 20S or 0.05µg 26S were used per reaction (rxn). **[B]** DmPI31 lacking the HbYX motif displays reduced ability to activate mammalian 26S proteasomes. These experiments were preformed as in [A] with 26S proteasomes. The K(apparent) was calculated by using the concentration of DmPI31 or DmPI31-HbYX at saturation.

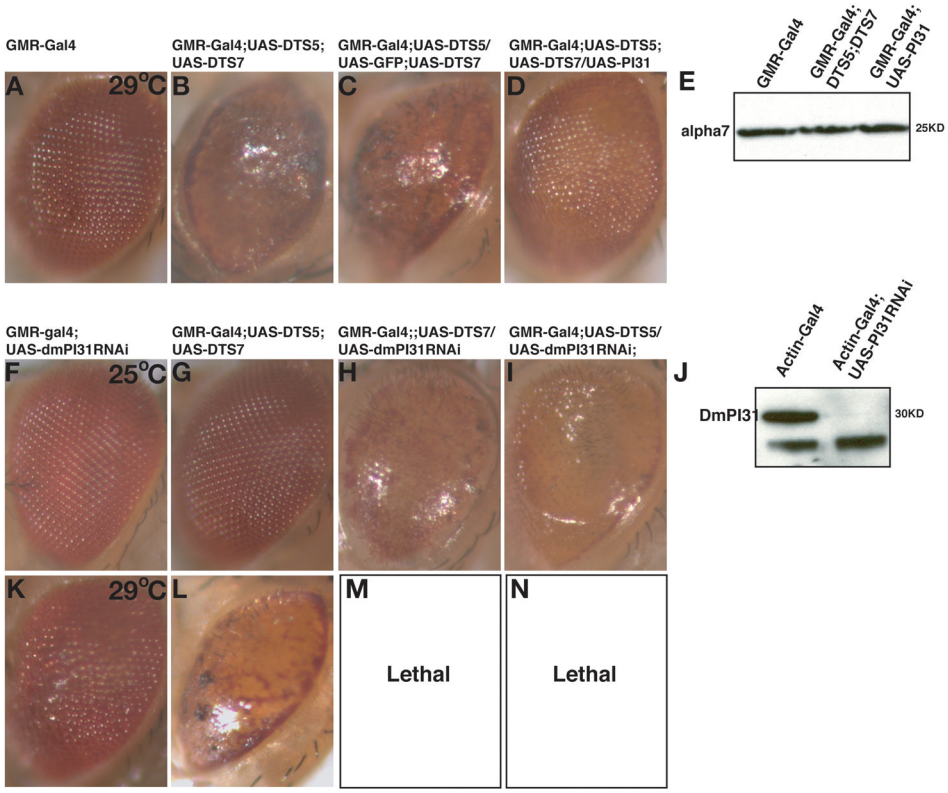

Figure 5. DmPI31 stimulates proteasome function *in vivo*

Inhibition of proteasome activity in the developing retina causes phenotypes that are sensitive to the amounts of DmPI31 protein. **[A-E]** Effects of expressing DmPI31 at 29°C. **[A]** GMR-Gal4 expression in a wild type background causes no detectable phenotype. **[B]** Inhibition of proteasome function upon expression of the dominant-negative temperature-sensitive mutants UAS-DTS5 and UAS-DTS7 in the developing retina at 29°C causes a small rough eye phenotype. **[C]** This phenotype is not affected by co-expressing GFP, but **[D]** is suppressed by co-expressing full-length DmPI31, indicating that elevated levels of DmPI31 can boost proteasome activity. **[E]** Western blot analysis of total eye lysates. The suppression of the rough eye phenotype is not caused by upregulation of the proteasome subunits, as verified by probing alpha7 subunit levels. **[F–N]** Depletion of DmPI31 enhances the eye phenotypes caused by reduced proteasome activity. Compared to flies expressing either *dmPI31*^{RNAi} **[F]** or the DTS proteasome mutants **[G]** at 25°C, combined expression of both *dmPI31*^{RNAi} and DTS proteasome mutant transgenes causes severe defects in eye development **[H–I]**. dmPI31^{RNAi} enhances the eye phenotypes of both DTS7 and DTS5 at 25°C. **[J]** Western blot demonstrating that the *dmPI31*^{RNAi} construct results in DmPI31 knock-down. **[K-N]** Expressing either DTS5 or DTS7 together with *dmPI31*^{RNAi} at 29°C, but not each alone, results in lethality. Structure-function studies of dmPI31 are summarized in Table S1.

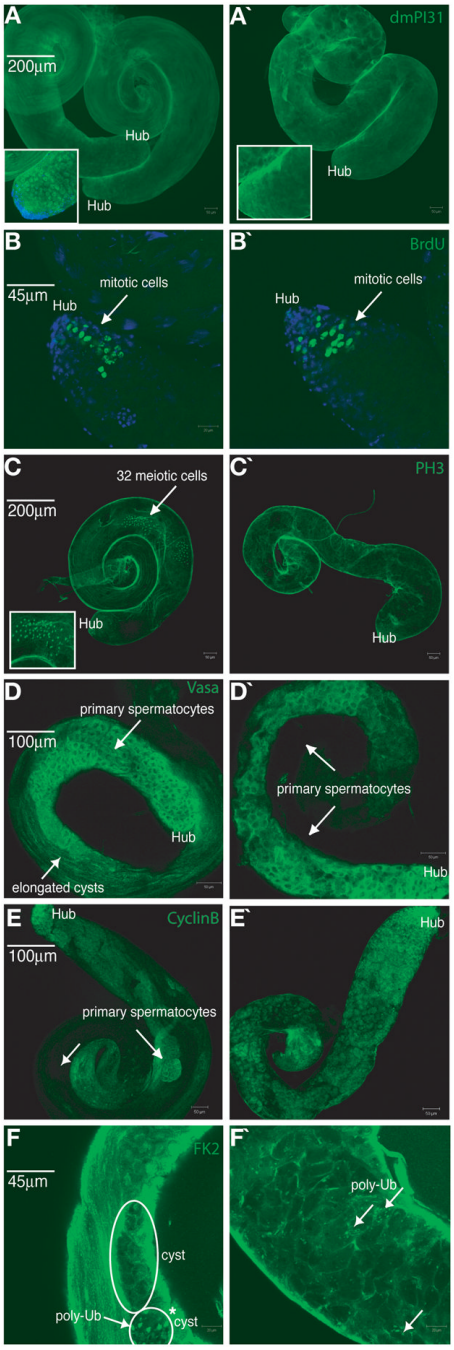

Figure 6. DmPI31 has an essential physiological function and is required for normal proteasome activity *in vivo*

[A–F] *dmPI31* mutants are lethal, but transgenic expression of DmPI31 in the soma rescues this lethality and permits the recovery of adult flies that lack DmPI31 in germ cells. These flies display defects in the cell-cycle and protein degradation. The left panel depicts wild-type testes, while the right depicts *dmPI31* mutants that have been rescued to adulthood. **[A–A′]** Distribution of DmPI31 protein in the testis. Testes were stained with and antibody staining towards DmPI31 (green). The wild type testis displays staining in germ-line stem cells (GSCs) and primary spermatocytes. This staining is lost in *dmPI31* mutant testes. The inset depicts a larger magnification of the apical tip, where these cells reside. **[B–B′]** BrdU

incorporation assay to detect dividing GSCs. Anti-BrdU staining (green) labeling S-phase cells is detected at the apical tip of the wild type testis. A similar labeling is detected in the *dmPI31* mutant testis, indicating that mitotic divisions are not disrupted. Nuclei are stained with DAPI (blue). **[C–C′]** Phospho-histone3 (PH3) antibody staining (green), which marks meiotic divisions. The PH3 antibody stains dividing nuclei, and therefore detects cells that are undergoing either mitosis or meiosis. Compared to wild-type testis, which display staining in the nuclei of 32 cell cysts, no staining is detected in cysts of *dmPI31* mutant testis, suggesting that meiosis is stunted. An arrow points to a 32-cell cyst (enlarged in inset) where PH3 staining is detected in wild type. **[D–D′]** Anti-Vasa antibody staining (green). This antibody is specific for germ cell progenitors. During normal differentiation, Vasa staining is strongest in GSCs and primary spermatocytes, and disappears when cells approach meiosis. In contrast, *dmPI31* mutant testes contain cysts with persistent Vasa staining, indicating that these cells maintain progenitor identity and fail to differentiate. **[E–E′]** CyclinB staining. CyclinB is normally detected in 16 cell stage cysts and disappears just before meiosis completes. In *dmPI31* mutant cells, CyclinB persists in 16-cell stage cysts, indicating that the degradation of this protein does not occur normally. See also Figure S4. **[F–F′]** FK2 staining of ubiquitin-conjugated proteins. FK2 detects the abundance of poly-ubiquitnated proteins and is thus a detector of proteasome activity. In normal germ-line cells (a pre-meiotic cyst is circled), FK2 staining is diffuse, and prominent staining is detected in differentiating nuclei (asterisks in separate cyst). In contrast, most *dmPI31* mutant cells display punctate staining (arrows point to poly-ubiquitnated protein clusters), indicating accumulation of non-degraded poly-ubiquitinated proteins.

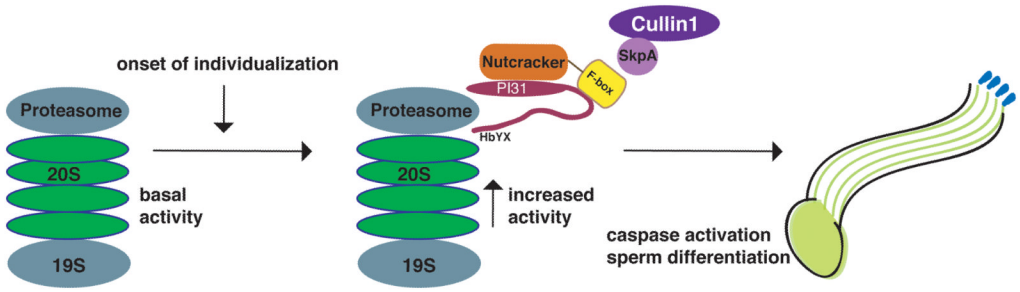

Figure 7. Model for proteasome regulation by the Nutcracker-DmPI31 complex

During their terminal differentiation, spermatids undergo a severe reduction in cell volume. This process, termed “individualization”, requires proteasome activity (Zhong and Belote, 2007). Both Nutcracker and DmPI31 are required for normal proteasome activity in the testis (Bader et al., 2010). The binding of Nutcracker stabilizes DmPI31 by protecting the carboxy-terminal region of DmPI31 from cleavage. The C-terminal domain of DmPI31 is necessary for both binding to proteasomes and to stimulate their activity (McCutchen-Maloney et al., 2000). This domain contains a HbYX motif that has been implicated in proteasome gate opening (Smith et al., 2007). The formation and binding of the DmPI31-F-box regulatory complex stimulates proteasome activity and promotes caspase activation and spermatid differentiation. Since the function of Nutcracker is restricted to the testis, whereas DmPI31 has an essential function in somatic cells, it is likely that other factors contribute to the regulation of DmPI31.

Table 1

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