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A novel F-box protein is required for caspase activation during cellular remodeling in *Drosophila*

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SUMMARY

Terminal differentiation of male germ cells in *Drosophila* and mammals requires extensive cytoarchitectural remodeling, the elimination of many organelles, and a large reduction in cell volume. The associated process, termed spermatid individualization, is facilitated by the apoptotic machinery, including caspases, but does not result in cell death. From a screen for genes defective in caspase activation in this system, we isolated a novel F-box protein, which we termed Nutcracker, that is strictly required for caspase activation and sperm differentiation. Nutcracker interacts through its F-box domain with members of a Cullin-1-based ubiquitin ligase complex (SCF): Cullin-1 and SkpA. This ubiquitin ligase does not regulate the stability of the caspase inhibitors DIAP1 and DIAP2, but physically binds Bruce, a BIR-containing giant protein involved in apoptosis regulation. Furthermore, *nutcracker* mutants disrupt proteasome activity without affecting their distribution. These findings define a new SCF complex required for caspase activation during sperm differentiation and highlight the role of regulated proteolysis during this process.

KEY WORDS: Caspase, SCF, Ubiquitin, IAP, Proteasome, Spermatogenesis, *Drosophila*

INTRODUCTION

Caspases are a family of cysteine proteases that are responsible for executing apoptosis, a form of programmed cell death that is essential for metazoan development and organismal homeostasis (Abraham and Shaham, 2004; Steller, 1995; Steller, 2008). Because of the inherent destructive function of these proteases, caspase activities are tightly regulated by both activators and inhibitors. Abnormal regulation of caspases is a hallmark of several diseases, including many types of cancer (Hanahan and Weinberg, 2000; Reed, 2003; Thompson, 1995; Vucic, 2008). The first level of caspase regulation is intrinsic to caspases themselves. Caspases are expressed as inactive zymogens that are cleaved to form the active enzyme, a step that is highly regulated, as there are multiple signaling pathways that govern their activation (Kornbluth and White, 2005). Furthermore, once caspases are activated by cleavage, their activity is held in check by a family of inhibitors, called inhibitor of apoptosis proteins (IAPs) (Deveraux and Reed, 1999; Salvesen and Duckett, 2002). These conserved proteins bind the active site of caspases through their baculovirus inhibitory repeat (BIR) domain, and only when they are removed can caspase substrates gain access to the protease (Hinds et al., 1999).

Post-translational modifications are another way by which caspases are regulated. The balance between pro- and anti-apoptotic factors is controlled by ubiquitin-dependent degradation, as well as by non-degradative ubiquitin modifications (Bader and Steller, 2009; Bergmann, 2010; Broemer and Meier, 2009; Vaux and Silke, 2005; Zhong and Belote, 2007). Some IAPs contain a RING domain that can act as a ubiquitin ligase, and the balance between their self- and caspase ubiquitylation is a major determinant of their activity (Ditzel et al., 2008; Lisi et al., 2000; Ryoo et al., 2002; Schile et al., 2008; Wilson et al., 2002; Yang et al., 2000).

An added level of complexity to caspase regulation is their non-apoptotic role in certain cells. Caspases are involved in diverse vital processes, including cellular signaling, differentiation and remodeling (Feinstein-Rotkopf and Arama, 2009; Kuranaga and Miura, 2007; Yi and Yuan, 2009). This type of activity requires very sensitive and nuanced control, as unrestrained caspase activity is destructive. For example, non-lethal caspase activity facilitates axonal branch removal during normal neuronal development in flies and mammals (Kuo et al., 2006; Nikolaev et al., 2009; Williams et al., 2006). However, it is thought to contribute to neurodegeneration when misregulated in Alzheimer's disease (Nikolaev et al., 2009).

In *Drosophila*, non-apoptotic caspase activation occurs at the final stage of sperm differentiation, also called individualization (Arama et al., 2003; Arama et al., 2007; Arama et al., 2006; Muro et al., 2006). This process is reminiscent of apoptosis in the sense that most of the cytoplasmic contents are exterminated to create highly motile sperm (Fuller, 1993; Tokuyasu et al., 1972) (Fig. 1A). Apoptotic regulators, such as Cytochrome c, Ark (the *Drosophila* homolog of Apaf-1), the initiator caspase Dronc (Nedd2-like caspase – FlyBase), and the IAP antagonist Hid (Wrinkled – FlyBase) have all been implicated in this process, suggesting that the basic molecular mechanisms of caspase activation are also conserved between this process and conventional apoptosis (Arama et al., 2003; Arama et al., 2006; Huh et al., 2004). A screen for genes that regulate caspase activation in this system uncovered a novel Cullin-3-based complex (Arama et al., 2007). Significantly, Cullin-3 was also recently implicated in the regulation of caspase-8 activation in mammals (Jin et al., 2009). Therefore, the isolation of additional genes from this screen is a promising avenue of research to identify novel players and/or pathways involved in the regulation of caspase activation.

Here, we describe a new ubiquitin ligase complex that regulates caspase activity during sperm individualization. From the genetic screen, we isolated an F-box protein, which we called Nutcracker, that shares some sequence similarity with the mammalian FBXO7

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protein. Flies mutant for *nutcracker* are viable but male sterile, displaying elongated spermatids, severe individualization defects and an absence of activated caspases. We further show that Nutcracker physically interacts with SkpA and Cullin-1 (Lin-19-like – FlyBase), which are two main components of a Skp/Cullin/F-box (SCF) ubiquitin ligase complex, and that the F-box domain of Nutcracker is important for both complex binding and caspase activation. Nutcracker can also associate with Bruce, a giant BIR and UBC domain-containing IAP-like protein, indicating a direct link between this SCF complex and the apoptotic machinery. Finally, we show that loss of *nutcracker* function causes reduced proteasome activity without affecting their distribution or numbers. These findings are the first to demonstrate the role of an SCF ubiquitin ligase complex in caspase activation and proteasome regulation, and they suggest that the involvement of controlled proteolysis in caspase activation is broader than has been previously appreciated.

MATERIALS AND METHODS

Fly strains

yw flies were used as wild-type controls. The Zuker mutant Z3-4692 (*ms771*) was obtained from C. S. Zuker (UCSD); alpha6T;Alpha6T-GFP was obtained from John Belote (Syracuse University); the deficiency lines *Df(3L)HR119* and *Df(3L)GN34* and the PBac insertion *PBac{WH}CG10855^{f07259}* from the Bloomington Stock Center; and *Df(3L)Exel6097* from Exelixis.

Genetic screen of the Zuker male-sterile collection lines

For technical details of the screen, see Arama et al., supplementary 4 (Arama et al., 2006).

Electron microscopy

Testes were sectioned and imaged as described (Arama et al., 2006).

Molecular biology

Drosophila Genomics Resource Center (DGRC) clone LD12948 was used to amplify the *CG10855* (*nutcracker*) ORF. For rescuing the *nutcracker^{ms771}* mutant phenotypes, the *CG10855* ORF was amplified by PCR (primers 1 and 2, see Table S1 in the supplementary material) and cloned into pHSP83(5′-3′UTRs) (Arama et al., 2006), which contained the 5′ and 3′ UTRs of *Cytochrome c distal* (*Cyt-c-d*). For antibody generation, the *CG10855* ORF was amplified by PCR (primers 3 and 4) and cloned into pET41a(+) (Novagen), which contains an N-terminal GST tag. For antibody affinity purification, the *CG10855* ORF was also cloned into pET101/D-TOPO (Invitrogen) (primers 5 and 6), which contains a C-terminal His tag. For testes expression of tagged proteins, a Protein A (PrA) tag was cloned into Casper-4 containing the *don juan* (DJ) promoter (primers 7 and 8), and full-length *CG10855* was cloned in frame with it (primers 9 and 10). ΔFbox was created by PCR amplification of *CG10855* without the last 174 nucleotides, and this product was also cloned in frame into Casper-4-DJ-PrA (primers 9 and 11). The construction of the *Bruce* ‘mini-gene’ was as detailed (Arama et al., 2007).

Antibody generation and tissue staining

Cleaved effector caspase antibody staining of young (0- to 2-day-old) adult testes was carried out as described (Arama et al., 2007), using a rabbit polyclonal anti-cleaved Caspase-3 (Asp175) antibody (Cell Signaling Technology) diluted 1:75. The only changes were that the subsequent TRITC-phalloidin (Sigma) incubation for staining of actin filaments was carried out during incubation with the secondary antibody, and that the slides were subsequently rinsed twice for 10 minutes each in PBS. Axonemal tubulin polyglycylation antibody staining was carried out using the mouse polyclonal antibody AXO 49 (a kind gift from Marie-Helene Bre, University of Paris-Sud, France) diluted 1:5000. Anti-CG10855 was created by injecting guinea-pig with full-length recombinant CG10855 (Cocalico Biologicals). CG10855 staining was carried out as described (Hime et al., 1996) using a 1:100 dilution of affinity-purified serum. The serum was purified by expressing a C-terminal His-tagged CG10855 in BL21 *E. coli* for 1.5 hours at 37°C. The bacterial lysate was run on SDS-PAGE, transferred onto an Immobilon-P membrane (Millipore) and blocked in 5% dried milk powder in PBST (0.1% Triton X-100 in PBS). Serum (500 μl) was pipetted onto the membrane and incubated for 3 hours, and, once removed, the purified antibody was eluted by 50 mM glycine (pH 2.5); the antibody was then neutralized with 0.1 M Tris (pH 9.0).

Genomic DNA Isolation and sequencing of the mutant alleles

Genomic DNA was isolated as described (Arama et al., 2007). This DNA (20 ng) was used to amplify *CG10855* with primers corresponding to its 5′ and 3′ UTRs (primers 12-15, see Table S1 in the supplementary material). PCR reactions were carried out using DyNAzyme EXT DNA polymerase (NEB) according to the manufacturer’s protocol. The products were purified using the High Pure PCR Product Purification Kit (Roche), concentrated by evaporation, and sequenced in a GENEWIZ sequencing facility.

RNA isolation and RT-PCR

Total RNA isolation and RT-PCR were conducted as described (Arama et al., 2007). Primers corresponding to the 5′ and 3′ UTRs (primers 12 and 13, see Table S1 in the supplementary material) were used to amplify *CG10855* mRNA.

Western blot

To create testes lysate, testes were dissected in lysis buffer (20 mM Hepes pH 7.6, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM DTT), homogenized and spun at 14,000 rpm (20,800 ***g***) for 15 minutes at 4°C. Protein concentration was determined by Bradford assay (BioRad), and 30-50 μg total protein, dissolved in SDS loading buffer, was separated by SDS-PAGE. After transfer, the Immobilon-P membrane was blocked with 5% dried milk powder in TBST (0.1% Tween 20 in PBS) for 1 hour, and incubated with primary antibody overnight at 4°C. The membrane was then washed three times in TBST, incubated in HRP-conjugated secondary antibody for 1 hour, and washed three more times with TBST before developing with ECL reagents (Amersham) and exposure to Kodak Biomax MR film. The following antibodies were used at a 1:1000 dilution: anti-Nutcracker (serum), anti-Cullin-1 (Zymed), anti-SkpA (a kind gift from T. Murphy, Carnegie Institution of Washington, Baltimore, MD, USA), anti-DIAP1 (a kind gift from H. D. Ryoo, NYU Medical School, NYC, NY, USA), anti-DIAP2 (a kind gift from P. Meier, Institute of Cancer Research, London, UK) and anti-Bruce (Arama et al., 2007). Anti-Alpha7 (Biomol) was used at 1:200.

Immunoprecipitations

For testes immunoprecipitation (IP), testes were dissected in IP buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM DTT), lysed (as for western blot), and equal amounts of protein were incubated for 2 hours at 4°C with Dynabeads (Invitrogen) that had been conjugated to rabbit IgG according to the manufacturer’s protocol. The beads were washed five times in lysis buffer (except that detergent was reduced to 0.1%), and eluted in SDS loading buffer by boiling.

Proteasome activity assay

Testes were lysed as for western blot. Protein concentration was measured and equal amounts of lysates were added at a 1:1 volume ratio to the Proteasome-Glo Proteasome Activity Detection Kit (Promega). The assay was carried out according to the manufacturer’s protocol. All reactions were conducted in a 96-well plate and read on a SpectraMax M2 micro-plate reader (Molecular Devices).

RESULTS

A male-sterile mutant defective in caspase activation during sperm individualization

In order to identify novel genes involved in caspase activation during sperm differentiation, we conducted a screen for genes that control caspase activation in this system, as described (Arama et al., 2007). In brief, ~850 fly lines from the Zuker stock collection that

were previously shown to have individualization defects (Wakimoto et al., 2004) were screened for a lack of caspase activation in spermatids using anti-cleaved Caspase-3 antibody (Arama et al., 2003). This antibody detects the activity of *Drosophila* effector caspases such as Dcp-1 and Drice (Ice – FlyBase), but is also a marker for Caspase-9-like Dronc (nc – FlyBase) activity (Fan and Bergmann, 2009). Several complementation groups/genes were identified that displayed complete loss of caspase activation. To test whether the block in caspase activation is direct and not a consequence of premature developmental arrest of these mutant spermatids, we stained testes from these lines with the AXO 49 antibody, a late developmental marker that detects polyglycylation of axonemal tubulin, a modification that accumulates at the onset of individualization (Arama et al., 2007; Bre et al., 1996; Bre et al., 1998; Rogowski et al., 2009). One of these cleaved caspase-negative and polyglycylation-positive mutants, which is represented in this screen by a single allele termed *ms771*, was homozygote viable with no gross defects apart from male sterility (Fig. 1B). Consistent with the idea that this mutant is specific for caspase activation, ultrastructural analysis portrayed morphologically intact mitochondria and axoneme, although some vacuolar structures were detected, indicating individualization defects (Fig. 1C).

The *ms771* mutant maps to an uncharacterized F-box protein, *nutcracker*

To genetically map *ms771*, the deficiency 'kit' of large genomic deletion lines was screened for deletions that fail to complement its sterility. Two overlapping lines, *Df(3L)HR119* and *Df(3L)GN34*, corresponding to regions 63C2;63F7 and 63E8-9;64A8-9, respectively, failed to complement the sterility phenotype of *ms771*, and the transheterozygotes stained negative for active Caspase-3. A smaller deficiency in the overlapping region, *Df(3L)Exel6097* (Fig. 2A), also failed to complement sterility and caspase staining (Fig. 2B). This deficiency deletes 15 genes, and the coding regions of several of them were PCR amplified from *ms771* mutants and sequenced. A premature stop

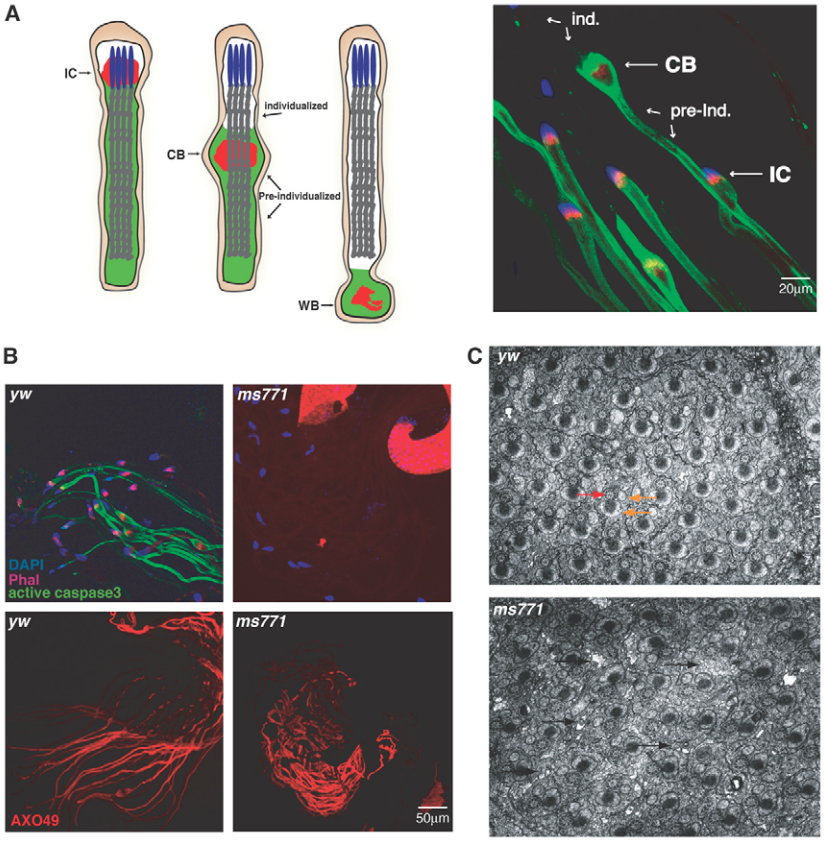

Fig. 1. *ms771* is a male-sterile mutant defective in caspase activity during sperm differentiation. (**A**) Diagram (left) and immunostaining (right) of spermatid individualization in *Drosophila*. At the final stage of differentiation, an actin-based individualization complex (IC) is formed around the elongated nuclei of 64 spermatids that are connected by cytoplasmic bridges. As the IC moves down the length of the spermatids, it expels the excess cytoplasm and unneeded organelles, leaving each spermatid engulfed in its own membrane. During this movement, the excess material accumulates around the IC to create the cystic bulge (CB). When the complex reaches the end of the tails, the CB turns into the waste bag (WB), which eventually degrades. In the right-hand panel, confocal images of several wild-type (*yw*) cysts illustrate the individualization (ind.) process. The cysts were stained with DAPI (nuclei, blue), phalloidin (IC, red) and for cleaved Caspase-3 (cytoplasm, green). (**B**) In contrast to cysts from *yw* testes, cysts from *ms771* homozygotes do not stain for cleaved Caspase-3. The nuclei (DAPI stained) in the mutant elongate, but the IC does not form. Like wild-type cysts, *ms771* cysts stain positively with AXO 49 antibody, a late differentiation marker, indicating that these deficiencies are not caused by a global differentiation defect. (**C**) Electron micrographs of cysts during individualization. Each spermatid within the cyst contains an axoneme (red arrow) and two mitochondrial derivatives (smaller and larger round structures, orange arrows). The *ms771* mutant displays normal formation of these structures, but the space between the spermatids indicates that the overall cyst structure preceding individualization is defective (black arrows).

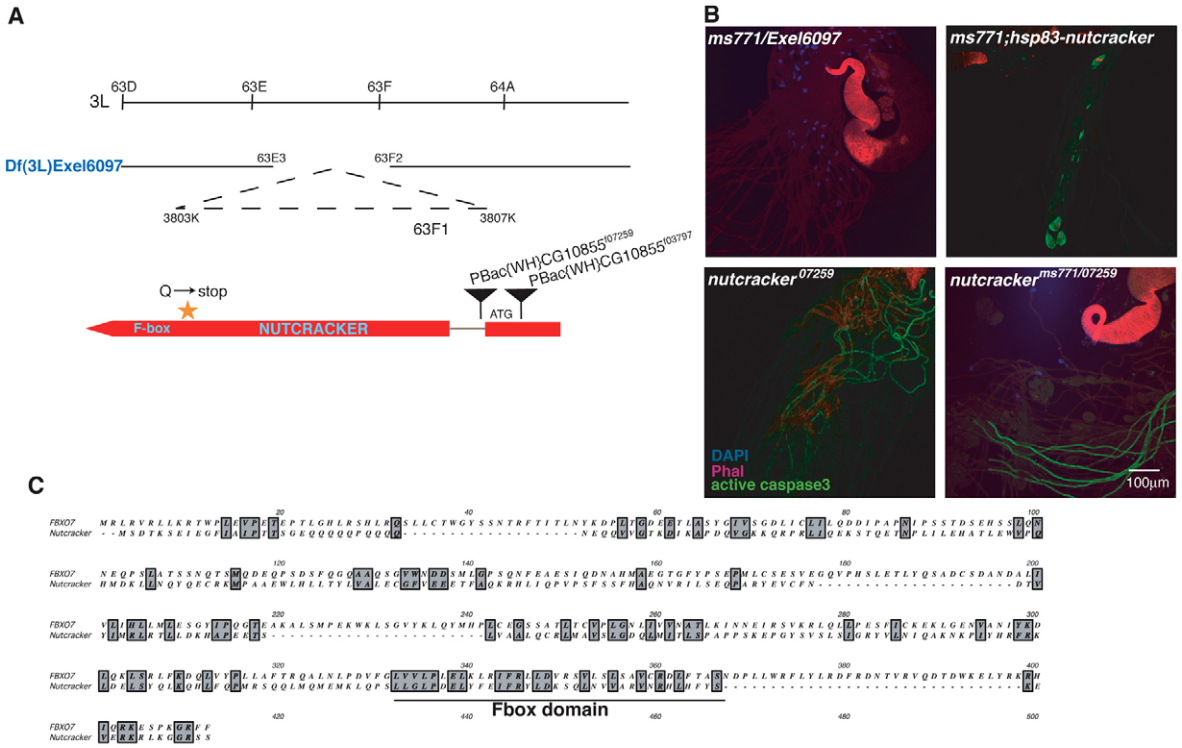

Fig. 2. *ms771* maps to *nutcracker* (*CG10855*), which encodes an uncharacterized F-box protein expressed in testes. (**A**) *nutcracker* genomic region. *nutcracker* is located at position 63F1, within the region removed by the deficiency *Df(3L)Exel6097*. Two piggyback insertions (inverted triangles) are annotated for *nutcracker*: *PBac{WH}CG10855^{f03797}* is inserted upstream of the ATG start site, and *PBac{WH}CG10855^{07259}* (*nutcracker^{07259}*) is inserted in the intronic region. An orange star indicates the location of the premature stop codon mutation in *nutcracker^{ms771}* that results in the truncation of the F-box domain. (**B**) The *ms771* mutation maps to *nutcracker*. The deficiency *Df(3L)Exel6097* fails to complement *ms771* sterility and staining, and both these defects are rescued by reintroducing *nutcracker* ORF. *nutcracker^{07259}* also has individualization defects and is hypomorphic for Caspase-3 staining. The staining is slightly reduced, but not eliminated, in the transheterozygote *nutcracker$^{ms771/07259}$*. (**C**) The F-box protein Nutcracker shares sequence homology with the F-box-only protein FBXO7. Alignment of the *Drosophila* Nutcracker protein against human FBXO7 (accession number CAG30377). The proteins exhibit highest similarity in their F-box domains. Overall, they share 17 identical amino acids (5% of Nutcracker, 2% of FBXO7), and 36 similar amino acids (11% of Nutcracker, 7% of FBXO7). Not shown are the last ~100 amino acids of FBXO7, as it is a longer protein.

codon mutation was identified in a novel gene (*CG10855*) that encodes a putative F-box protein. This uncharacterized F-box protein displays limited amino acid conservation with the mammalian F-box protein FBXO7, and in both proteins the F-box domain is uncommonly close to the C-terminus (Fig. 2C). We termed *CG10855 nutcracker,* for 'novel ubiquitin targeting complex required for activating caspases'.

We next looked for possible transposon-derived mutations in *nutcracker* and identified a *piggyBac* insertion in the intron of *nutcracker* (*nutcracker^{07259}*), which was obtained from the Bloomington Stock Center (Fig. 2A). Complementation analysis with the *nutcracker^{ms771}* mutant resulted in a failure of *nutcracker^{07259}* to complement the male sterility of the former, indicating that *nutcracker^{07259}* is indeed an allele of *nutcracker*. Staining of testes from the *nutcracker^{07259}* mutants revealed individualization defects, although they still exhibited some level of active caspases, suggesting that it is a weak allele of *nutcracker* (Fig. 2B). Finally, transgenic expression of an intact *nutcracker* gene using the testis-specific promoter of the *Hsp83* gene restored proper caspase activation, spermatid individualization and fertility in both *nutcracker* mutants, indicating that all the male sterility-associated phenotypes are due to mutations in *nutcracker* (Fig. 2B).

We also generated an antibody to full-length Nutcracker. A band of ~35 kDa, corresponding to the predicted size of Nutcracker, was detected in testes lysates by western blotting (Fig. 3A). By contrast, a smaller band was detected in lysates from *nutcracker^{ms771}* testes, consistent with the finding that this allele contains a premature stop codon that deletes the entire F-box domain (Fig. 3A). The fact that this stable, but truncated, protein still displays the mutant phenotypes suggests that the F-box domain is important for the physiological role of Nutcracker in sperm differentiation.

Analysis of *nutcracker^{07259}* mRNA by RT-PCR revealed a 600 bp insertion that causes a frame shift, which was likely to be a result of failed splicing due to the transposon insertion (Fig. 2A, Fig. 3B). This mutation results in complete elimination of Nutcracker protein as detected by western analysis (Fig. 3A). However, because this allele behaves as a genetic hypomorph, it is likely that a small amount of wild-type protein is still produced, but at levels below western blot detection.

To further characterize *nutcracker* expression, we first examined its mRNA distribution. We extracted mRNA from either wild-type or *son-of-oskar* [males born to *oskar* mutant females (Lehmann and Nusslein-Volhard, 1986; Arama et al., 2007)] males, which lack germ cells and functioning testes, and compared *nutcracker* levels by semi-

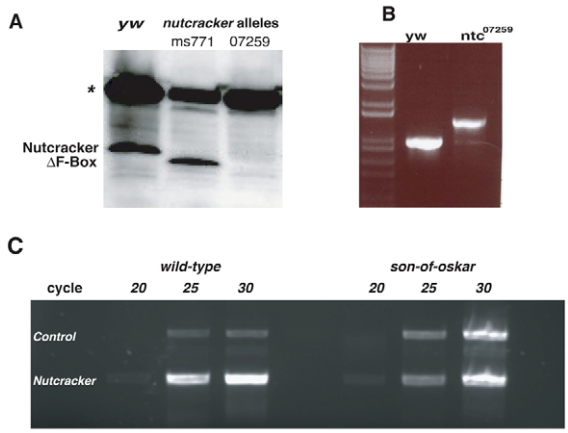

Fig. 3. *ms771* harbors a truncated Nutcracker protein. (**A**) Western blot of testes lysates using the Nutcracker antibody detects a band of the predicted size (~35 kDa). Analysis of testes lysates taken from the mutant flies indicate that whereas *nutcracker*^{ms771} (ms771) harbors a truncated form of Nutcracker protein, the *nutcracker*^{07259} (07259) mutant is a protein null. The asterisk indicates a non-specific band that serves as a loading control. (**B**) RT-PCR analysis of *nutcracker* mRNA transcripts collected from wild-type (*yw*) or *nutcracker*^{07259} (*ntc*^{07259}) adult testes. Compared with the transcript of predicted size found in wild-type testis, the *nutcracker*^{07259} transcript contains a ~600 nt insertion. Sequencing analysis of the corresponding band revealed that this insertion is located in the middle of the transcript, resulting in a frame shift in the ORF. (**C**) *nutcracker* is expressed in the germ line. Semi-quantitative RT-PCR of *nutcracker* mRNA using total mRNA taken from either wild-type or *son-of-oskar* males. More transcripts were detected in the wild type, indicating that it is preferentially expressed in testes.

quantitative RT-PCR. This analysis revealed that more mRNA is found in the wild-type animal (cycle 25, Fig. 3C), indicating that *nutcracker* mRNA is preferentially expressed in testes.

Nutcracker is part of an SCF ubiquitin ligase complex

F-box-containing proteins can function as substrate-binding adaptors in SCF ubiquitin ligase complexes, which mediate the ubiquitylation of substrate proteins, thus targeting them for degradation by the 26S proteasome (Cardozo and Pagano, 2004; Kipreos and Pagano, 2000). These complexes comprise an Skp protein that links the F-box protein to the Cullin-1 scaffolding protein. To examine whether Nutcracker is in complex with other SCF members in the testis, we tagged Nutcracker with Protein A (PrA) and placed them downstream of the *don juan* (DJ) promoter and 5′UTR and upstream of the *Cyt-c-d* 3′UTR (DJ-PrA-ntc), which are specifically and highly expressed in spermatids (Arama et al., 2006; Santel et al., 1998; Santel et al., 1997). Transgenic fly lines were generated and checked for expression by western blotting (not shown). These transgenes restored caspase staining, but failed to rescue the sterility phenotype of the *nutcracker* mutants, possibly owing to misexpression of this construct or domain obstruction by the PrA tag. Co-immunoprecipitation (co-IP) experiments using lysates of testes from these transgenic flies showed that PrA-ntc specifically binds both endogenous SkpA and Cullin-1, suggesting that Nutcracker functions in an SCF complex (Fig. 4A). To investigate the role of the F-box domain in mediating this interaction, we also constructed a truncated form of PrA-tagged Nutcracker by deleting the F-box domain (PrA-ntcΔF), similar to the stop codon mutation in *nutcracker*^{ms771}. Unlike full-length Nutcracker, PrA-ntcΔF was unable to enrich Cullin-1 or SkpA in co-IP experiments (Fig. 4A). These results confirm that Nutcracker forms an SCF complex that is mediated through its F-box domain, and raises the possibility that the phenotypes observed *in nutcracker*^{ms771} are caused by the inability to form this complex.

Nutcracker and Cullin-1 colocalize with actin cones at the individualization complex

At the onset of individualization, actin organizes into cone structures that form around the elongated nuclei to create the individualization complex (IC) (Fig. 1A) (Noguchi and Miller, 2003). As the IC moves, it expels and ‘pushes out’ most of the cytoplasm and unneeded organelles, resulting in the formation of an inflated structure called the cystic bulge (CB) (Fuller, 1993). We visualized Nutcracker localization in the context of the morphological events during individualization. For immunostaining, we affinity purified Nutcracker serum and used it to stain testes (Fig. 4B). Nutcracker partially colocalized with the forming complex, although they did not completely overlap. As the complex moved, Nutcracker staining was detected within the CB, but the most prominent staining was observed around the outer layer of the IC itself, at the base of the cones. Various focal planes of the CB showed that the majority of the protein accumulates around the outer borders of the IC, and only slightly intertwines between the cones. This indicates that the protein might be anchored in the vicinity of the sites of cone attachment to the outer membrane. We also stained testes from *nutcracker*^{07279} flies, which failed to display a protein band by western blot. Similarly, we were unable to detect protein by immunostaining (Fig. 4B), demonstrating the specificity of the antibody in detecting Nutcracker.

We investigated whether this localization of Nutcracker corresponded to the localization of the SCF complex. The pattern of Cullin-1 staining at ICs in *yw* testes was similar to that of Nutcracker (Fig. 4C), placing these proteins in the same vicinity on the IC. Together with the biochemical data, this strongly suggests their physical interaction in vivo and their combined role in individualization.

nutcracker mutants display defects in individualization complex formation

nutcracker^{ms771} mutant testes are defective in IC formation, as no actin cone formation is observed around the elongated nuclei (Fig. 1B). When Nutcracker is misexpressed in this background, as in the DJ-PrA-ntc transgene, these phenotypes are partially rescued: flies displayed cleaved Caspase-3 staining and some formed ICs at the tip of the cyst (Fig. 4D). However, these ICs appeared irregular, were mostly scattered and, in some cases, individual actin cones could be observed scattered along the length of the cyst. This might explain why sterility is not rescued, and indicates that the timing and level of Nutcracker expression are important for IC integrity. Furthermore, in the testes of the hypomorphic allele *nutcracker*^{07259}, which stain positive for cleaved Caspase-3, some irregular ICs were observed (Fig. 4B), further suggesting that actin filament formation and organization and caspase activation are sensitive to the levels of Nutcracker.

Nutcracker physically interacts with Bruce, a giant IAP-like protein

In *Drosophila*, two IAPs have been shown to directly inhibit active caspases: DIAP1 and DIAP2 (Thread and Inhibitor of apoptosis 2 – FlyBase) (Hay et al., 1995); however, only DIAP1 is strictly needed

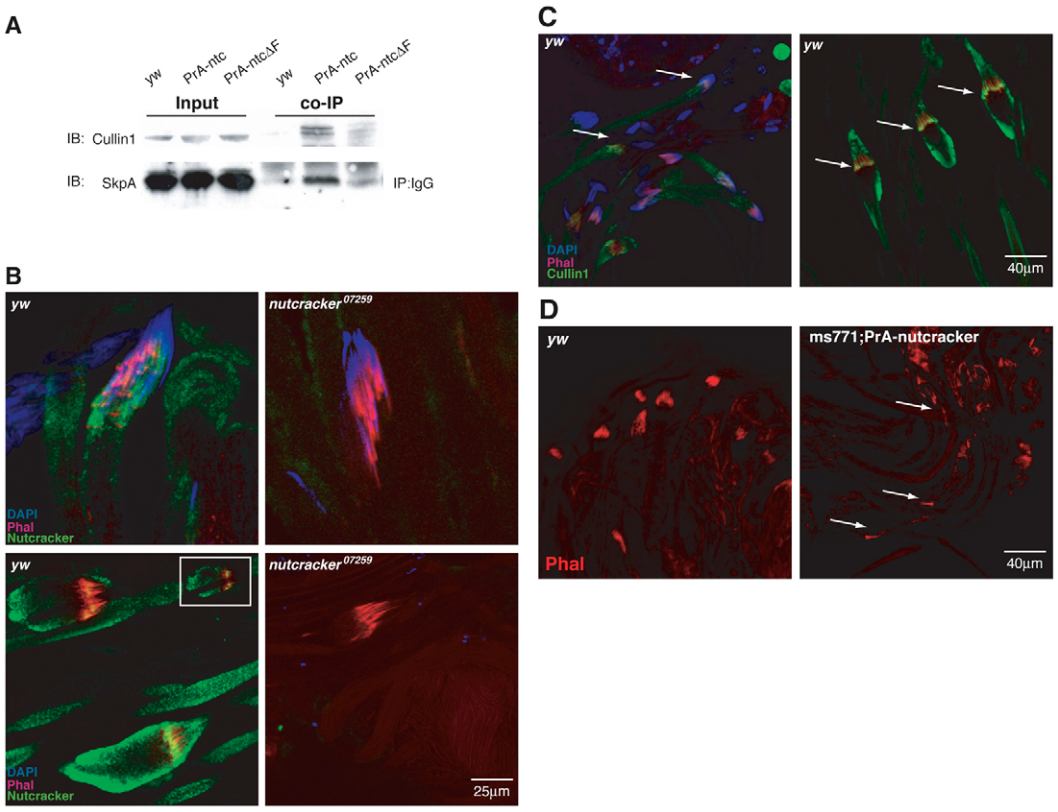

Fig. 4. Nutcracker and Cullin-1 form an SCF complex and colocalize with actin at the individualization complex. (**A**) The F-box protein Nutcracker co-immunoprecipitates (co-IPs) with members of the SCF complex. Protein A (PrA)-tagged Nutcracker (PrA-ntc) was expressed in testes and its endogenous interacting partners were identified by western blot. Co-IP followed by western blot analysis using either Cullin-1 or SkpA antibodies demonstrates that Nutcracker binds both Cullin-1 and SkpA, which are both part of the SCF ubiquitin ligase (the middle lane of the co-IP). The interaction is hindered by the removal of the F-box domain (PrA-ntcΔF), suggesting that Nutcracker is part of this complex (right-hand lane of the co-IP). The input panel indicates that equal amounts of either SkpA or Cullin-1 were present in the lysate at the beginning of the experiment. (**B**) Nutcracker antibody staining (green). As the IC forms around the elongated nuclei, Nutcracker protein accumulates around it (upper left). After the IC begin to move, Nutcracker localizes around the bulge, intertwined within the complex, as can be seen in the two planes shown (lower left). The staining completely disappears in *nutcracker*^{07259}, which occasionally displays formed (yet defective) ICs (right-hand panels). The ICs are stained with phalloidin (red), and the nuclei are stained with DAPI (blue). (**C**) Cullin-1 staining (green) of *yw* testes showing similar staining pattern to Nutcracker (arrows point to the staining around the nuclei in the left panel, and to the staining around the IC in the right panel). (**D**) Nutcracker misexpression affects IC integrity. DJ-PrA-ntc was expressed in the *nutcracker*^{ms771} background. The DJ promoter drives high levels of expression at later stages of differentiation, so Nutcracker is overexpressed and mistimed. This misexpression does not rescue sterility, but does restore some cleaved Caspase-3 staining (not shown). The IC, which does not form in *nutcracker*^{ms771}, does form in this background (phalloidin, red), but its formation is altered and the actin cones are scattered (white arrows).

for controlling caspases in vivo (Goyal et al., 2000; Lisi et al., 2000). IAPs are regulated by ubiquitin-mediated degradation, which removes them from caspases, thus allowing proteolysis (Ryoo et al., 2002; Wilson et al., 2002). We examined whether a Nutcracker-containing SCF ubiquitin ligase mediates the degradation of these proteins. We checked steady-state levels of DIAP1 protein in *nutcracker*^{ms771} mutants by western blot and found no differences with the wild type, suggesting that Nutcracker does not regulate DIAP1 stability (Fig. 5A). Furthermore, co-IP experiments using testes lysates, and those conducted by co-expressing tagged DIAP1 and Nutcracker proteins in S2 cells, failed to detect an interaction between these proteins (data not shown). We also checked DIAP2, which is able to inhibit caspases in vitro (Ribeiro et al., 2007) and has a role in *Drosophila* immunity (Kleino et al., 2005). The levels of this inhibitor were also unchanged in *nutcracker*^{ms771} mutants (Fig. 5A). These results suggest that Nutcracker regulates caspase activation independently of the known direct inhibitors of caspases.

Next, we investigated another IAP, Bruce, which has a known role in spermatogenesis (Arama et al., 2003) and has been shown to inhibit apoptosis induced by Reaper (Vernooy et al., 2002). Bruce is a large (~500 kDa) protein that contains both a BIR domain and the UBC domain found in E2 conjugating enzymes. Mutations in *Bruce* are male sterile and cause nuclear degeneration, presumably by excess caspase activation (Arama et al., 2003). To check whether this protein is a potential substrate of Nutcracker, we conducted co-IP experiments by co-expressing PrA-ntc and a shorter version of Bruce (consisting of about half of the protein, including the BIR and UBC domains, as the entire gene is yet to be cloned) in S2 cells. In this experiment, the *Bruce* 'mini-gene' product physically interacted with both the full-length and truncated forms of Nutcracker (Fig. 5B).

In order to define the genetic interaction between *nutcracker* and *Bruce*, we generated double mutants by recombining *Bruce* mutants onto the *nutcracker*^{07259} chromosome. We rationalized

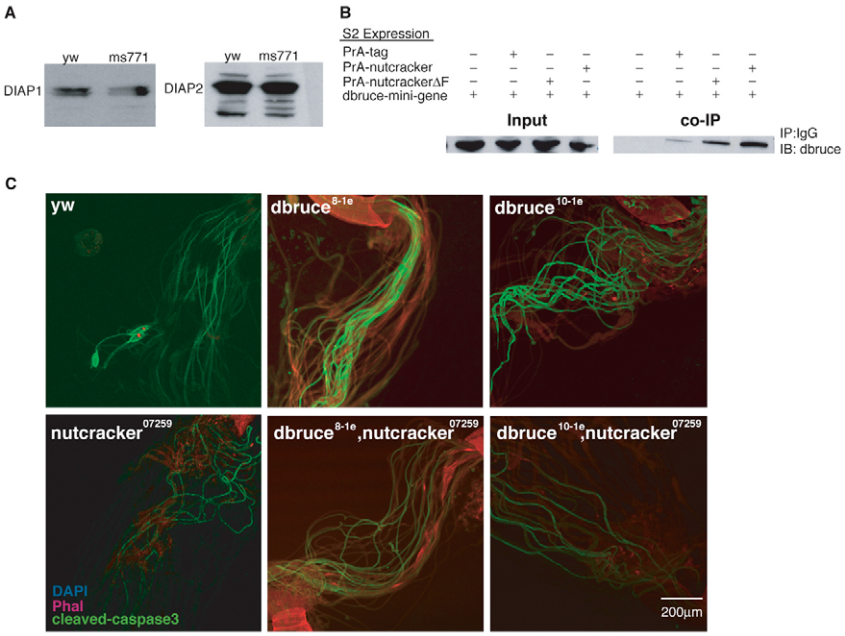

Fig. 5. Nutcracker physically interacts with Bruce, a giant IAP-like protein. (**A**) Western blots of total protein lysates from *yw* or *nutcracker*^{ms771} testes using either DIAP1 or DIAP2 antibodies indicate that Nutcracker does not affect the levels of these direct caspase inhibitors. (**B**) The F-box protein Nutcracker physically binds Bruce. PrA, PrA-ntc or PrA-ntcΔF were expressed in S2 cells together with the *Bruce* 'mini-gene' construct. Western blot analysis of this co-IP experiment revealed that Bruce binds both full-length and truncated Nutcracker, suggesting that Bruce is an interacting protein but that its interaction with Nutcracker is independent of the SCF complex. (**C**) Genetic interaction between *Bruce* and *nutcracker*. Caspase staining (green) of the hypomorphic allele *nutcracker*^{07259} is unchanged in double mutants with *Bruce*$^{8\text{-}1e}$ or *Bruce*$^{10\text{-}1e}$.

that if Bruce is the substrate of Nutcracker, then the decreased amount of caspase staining in this hypomorphic *nutcracker* mutant might be elevated in double mutants. We used two *Bruce* mutants previously isolated in our laboratory (J. Agapite, K. McCall and H.S., unpublished) (Arama et al., 2003). One mutant, 8-1e, was characterized as harboring a deletion of the BIR domain of Bruce, whereas the other, 10-1e, contains a deletion of almost the entire protein, including the UBC domain (but still contains the N-terminal BIR domain). When we stained the double mutants *Bruce*$^{8\text{-}1e}$*, nutcracker*^{07259} or *Bruce*$^{10\text{-}1e}$*, nutcracker*^{07259} we saw no change in caspase staining (Fig. 5C) as compared with the *nutcracker* single mutant. Furthermore, morphological analysis of either double mutant did not reveal more severe individualization defects than with single mutants. These data argue against a simple linear pathway in which Nutcracker activates caspases by inhibiting Bruce.

Proteasome activity is reduced in *nutcracker* mutants

Proteasome activity is important for spermatid individualization (Zhong and Belote, 2007). In particular, IC movement and caspase activation are abnormal in a mutant for a testis-specific proteasome subunit, *alpha6T* (*Prosα6T* – FlyBase). Given that some aspects of the *nutcracker* mutant phenotypes resemble the loss of *alpha6T* function, we explored whether *nutcracker* has an effect on proteasomes.

We utilized a GFP-tagged Alpha6T protein (Zhong and Belote, 2007) to ask whether proteasome distribution and numbers are altered in a *nutcracker* mutant background. Alpha6T-GFP is a faithful reporter of proteasome localization, as it can rescue *alpha6T* mutant phenotypes and is fully incorporated into proteasomes (Zhong and Belote, 2007). Alpha6T colocalized with the elongated nuclei just before IC formation (Fig. 6A, top right), but moved ahead of the actin cones after the IC forms (Fig. 6A, bottom right). In *nutcracker*^{ms771}, in which no ICs form, Alpha6T-GFP persisted in the nuclei (Fig. 6A, top left). By contrast, in the hypomorphic *nutcracker*^{07259}, in which occasional ICs form, Alpha6T-GFP was detected at its normal location ahead of the actin cones (Fig. 6A, bottom left). These data indicate that the movement and overall distribution of proteasomes do not depend on *nutcracker* function, but on IC formation.

We next asked whether *nutcracker* influences proteasome activity directly. We assayed proteasome activity in wild-type, *nutcracker*^{ms771} or *nutcracker*^{ms771} flies that express a *nutcracker* rescue construct. Testes from these genotypes were lysed and differences in proteasome activity were detected by measuring the hydrolysis of a fluorogenic peptide. Compared with wild-type testes, proteasome activity was reduced in *nutcracker*^{ms771} mutants (Fig. 6B). This activity was restored by reintroducing *nutcracker* into the mutant background, indicating that the decreased activity is indeed caused by reduced *nutcracker* function. Furthermore, in order to investigate whether this reduced activity is caused by a reduction in proteasome numbers in the mutant, we looked at the levels of three different proteasome subunits. Assaying individual subunit proteins is a good indicator of proteasome number because the majority of proteasome subunits in the cell are incorporated into the proteasome complex (Glickman and Raveh, 2005). Compared with the wild type, the levels of Alpha7 (Prosα7 – FlyBase), Rpn3 and Rpt4 were unchanged in *nutcracker*^{ms771} mutant testes (Alpha7 is shown in Fig. 6C; data not shown). These data demonstrate that equal numbers of proteasomes are formed in the mutant, suggesting that *nutcracker* acts directly on proteasome activity. Taken together, these results indicate that *nutcracker* controls caspase activation and spermatid individualization by regulating proteasome activity.

DISCUSSION

Nutcracker is a novel F-box protein that controls caspase activation

In this work, we have studied the regulation of non-apoptotic caspase activation during *Drosophila* spermatogenesis in order to understand the role of controlled proteolysis during differentiation and cellular remodeling. We identified a new component of this regulatory pathway – a novel F-box protein that we termed Nutcracker. Nutcracker is strictly required for caspase activation during spermatogenesis. The fact that Nutcracker can form a complex with bone fide components of an SCF ubiquitin ligase in

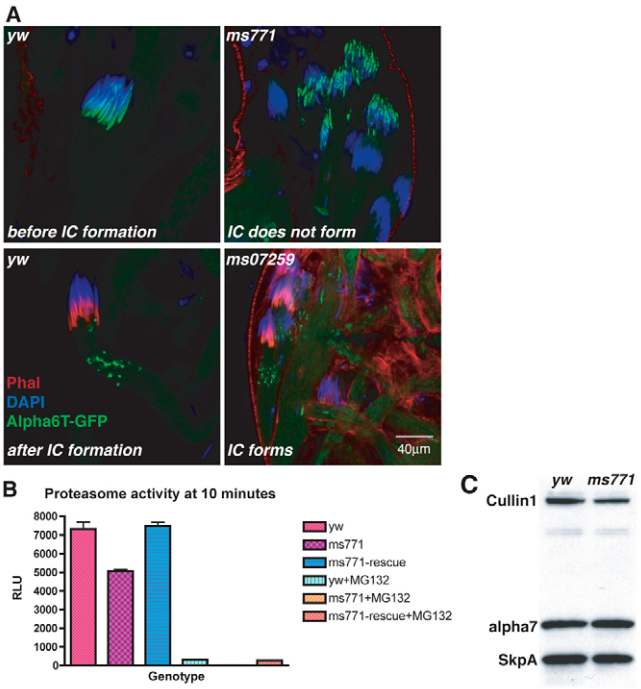

Fig. 6. Nutcracker affects proteasome activity but not distribution or numbers. (**A**) Proteasome distribution during individualization. GFP-tagged Alpha6T is a marker for proteasome localization (Zhong and Belote, 2007). Alpha6T is detected in elongated nuclei before the IC forms (top left), and after IC formation moves to the base of it (bottom left). Testes of *nutcracker*^{ms771} display no Alpha6T outside the nuclei (top right). By contrast, in the *nutcracker*^{07259} hypomorphic allele, in which occasional ICs are formed, Alpha6T is detected at its normal location ahead of the IC (bottom right). (**B**) Testes from *nutcracker*^{ms771} display decreased proteasome activity. Total protein was extracted from the indicated genotype and proteasome activity detected by measuring fluorogenic peptide hydrolysis after 10 minutes. Measurements are plotted as relative luminescence units (RLU). Each genotype sample was split and a proteasome inhibitor (MG132) was added to half the sample to confirm the specificity of the read-out. Wild type, *yw*; *nutcracker*^{ms771}, ms771; *nutcracker*^{ms771};*Hsp83-nutcracker*, ms771-rescue. (**C**) The levels of the proteasome subunit Alpha7 are unchanged in the *nutcracker*^{ms771} mutant. Total protein was extracted from either wild-type (*yw*) or *nutcracker*^{ms771} (*ms771*) testes and the amounts of the indicated proteins were determined by western blot analysis. Amounts of other SCF members (Cullin-1 and SkpA) were also assayed and used as loading controls.

the testis and that a mutant with a deleted F-box domain abrogates both the formation of this complex and caspase activation in spermatids, strongly suggest that the role of Nutcracker in spermatids is intimately associated with this SCF complex.

Most F-box proteins also possess another protein-interaction domain, usually comprising WD40 or LRR motifs, that is responsible for binding the ubiquitylation substrate (Cardozo and Pagano, 2004; Kipreos and Pagano, 2000). Nutcracker belongs to the class of F-box proteins that do not contain a known protein-protein interaction domain, and differs topologically from most F-box proteins in that its F-box domain is at the very C-terminus (Kirk et al., 2008). Sequence alignments with several F-box-only proteins revealed that Nutcracker shares some limited amino acid similarity with the mammalian FBXO7 protein, which also contains the F-box domain at the C-terminus (Fig. 2C). Although the sequence conservation is limited primarily to the F-box domain, it is possible that these two proteins share functional properties, as do other proteins that are conserved only within limited regions. For example, the *C. elegans* p53 protein displays less than 20% overall primary sequence similarity to the human protein, mostly in the active sites, but has been demonstrated to function in related cellular processes (Derry et al., 2001; Schumacher et al., 2001). Since FBXO7 has been shown to regulate the stability of cIAP1 (BIRC2) (Chang et al., 2006), it is possible that these two E3 ligases have a conserved function in caspase regulation.

The ubiquitin-proteasome system is implicated in regulating caspase activity. Several studies have shown that the ubiquitylation and degradation of DIAP1 is a means of displacing it from caspases when apoptosis is favored (Bader and Steller, 2009). Also, ubiquitylation of caspases themselves contributes to their regulation by preventing a critical mass of full-length caspases from auto-activation in a normal setting (Ditzel et al., 2008; Schile et al., 2008). The wide variety of other ubiquitin-modifying proteins that regulate apoptosis and caspase activity, including Bruce (Vernooy et al., 2002), Morgue (Hays et al., 2002; Schreader et al., 2003; Wing et al., 2002) and Uba1 (Lee et al., 2008), imply the existence of an elaborate regulatory network that is controlled by ubiquitylation.

In our screen we isolated another ubiquitin ligase, a Cullin-3-based complex, which indicates that caspase activation in this system is tightly controlled by ubiquitin modifications. These two complexes could regulate the stability of the same substrate, as is the case for regulation of Cubitus interruptus (Ci) stability in Hedgehog signaling by both Cullin-1-based and Cullin-3-based complexes (Jiang, 2006; Ou et al., 2002), or they might target multiple important substrates. Alternatively, the E3 ligases isolated in our screen might play non-degradative roles in controlling caspase activity. For example, mono-ubiquitylation affects the targeted localization of proteins (Haglund and Dikic, 2005) and these ubiquitin ligases might control the proper localization of caspase regulators. Another possibility is that these E3 ligases mediate the non-classical Lys63 ubiquitin chain addition that is important for protein-protein interaction. Thus, instead of degradation, these proteins might actually control interactions between caspase regulators.

Nutcracker binds the IAP Bruce

Although DIAP1 is the only *Drosophila* BIR-containing protein that has been shown to directly inhibit caspases in vivo, Bruce has been implicated in modifying apoptosis in several death paradigms, and mutations in its mammalian homolog cause defects associated with excess cell death (Bartke et al., 2004; Hao et al., 2004; Lotz et al., 2004). Here, we show that Bruce can physically bind Nutcracker, and that this interaction is independent of the F-box domain. Therefore, Bruce might be a substrate of Nutcracker. However, we were unable to determine the steady-state levels of Bruce in *nutcracker* mutants, so it is as yet unclear whether it is indeed a substrate or a complex partner. The fact that Bruce also binds to another E3 ligase isolated in our screen (Arama et al., 2007) suggests that this protein is a common regulator of caspase activation during individualization.

Nutcracker affects proteasome activity

We show that *nutcracker* mutants cause a reduction in proteasome activity. This decreased activity does not seem to be due to proteasome mislocalization or a reduction in their numbers, suggesting that Nutcracker controls proteasome activity directly. It

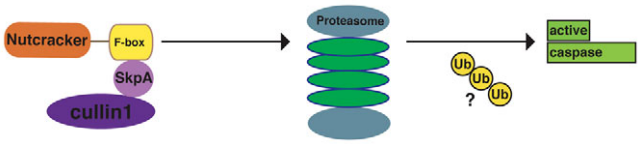

Fig. 7. A model for caspase activation by the *Drosophila* Nutcracker SCF complex. Our model suggests that Nutcracker forms a complex with members of an SCF ubiquitin ligase complex. This complex can then regulate caspase activation, possibly by modifying proteins that activate the proteasome; these could include, for example, components of the proteasome regulatory particle or other associated proteins. Caspase activation could then be facilitated by ubiquitin-mediated regulation.

is possible that Nutcracker modifies proteasome regulators, which could include, for example, proteins of the regulatory particle of the proteasome. An attractive model is that Nutcracker functions through proteasomes to activate caspases (Fig. 7). As mentioned above, caspase activity is tightly controlled by the ubiquitin proteasome system (Bader and Steller, 2009; Broemer and Meier, 2009). Therefore, it is possible that local activation of proteasomes controls localized caspase activation.

Many questions remain regarding the non-lethal role of caspases in cellular remodeling. For instance, is it a specialized activation that is governed by dedicated proteins, and to what extent are known apoptotic regulators involved in this process? Another intriguing question is how cells tolerate a certain level of caspase activation and avoid destruction by these potentially deadly proteases. Answers to these questions will not only uncover novel caspase regulators, but might also help us to understand how diseased cells, such as cancer cells, manage to escape cell death.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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