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; Broer 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 spermat 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 spermatid individualization. From the genetic screen, we isolated an F-box protein, which we called Nutcracker, that shares some sequence similarity with the mammalian FBXO7.
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); alphaT;AlphaT-GFP was obtained from John Belote (Yale University); the deficiency lines Df(3L)HR119 and Df(3L)GN34 and the PBac insertion PBac(WH)CG10855 from the Bloomingon Stock Center; and Df(3L)Excel6097 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 Genomes Resource Center (DGRC) clone LD12948 was used to amplify the CG10855 (nutcracker) ORF. For rescuing the nutcrackerms771 mutant phenotypes, the CG10855 ORF was amplified by PCR (primers 1 and 2, see Table S1 in the supplementary material) and cloned into pHS8355 (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 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 Heps 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 Heps pH 7.5, 150 mM NaCl, 15 mM MgCl2, 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.](image)
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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 is inserted upstream of the ATG start site, and PBac(WH)CG10855 (nutcracker07259) is inserted in the intronic region. An orange star indicates the location of the premature stop codon mutation in nutcracker07259 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. nutcracker07259 also has individualization defects and is hypomorphic for Caspase-3 staining. The staining is slightly reduced, but not eliminated, in the transheterozygote nutcracker07259. (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 (nutcracker07259), which was obtained from the Bloomington Stock Center (Fig. 2A). Complementation analysis with the nutcracker0771 mutant resulted in a failure of nutcracker07259 to complement the male sterility of the former, indicating that nutcracker07259 is indeed an allele of nutcracker. Staining of testes from the nutcracker0771 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 nutcracker0771 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 nutcracker07259 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-
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<sup>07259</sup> 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 <i>yw</i> 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 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 it downstream of the don Juan (DJ) promoter and 5' UTR and upstream of the <i>Cyt-c-d</i> 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<sup>ms771</sup>. 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<sup>ms771</sup> are caused by the inability to form this complex.

Nutcracker and Cullin-1 mutants display defects in individualization complex formation

nutcracker<sup>ms771</sup> 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<sup>07259</sup>, 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 <i>Drosophila</i>, 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...
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 nutcrackerms771 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 Droso phila immunity (Kleino et al., 2005). The levels of this inhibitor were also unchanged in nutcrackerms771 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 nutcracker07259 chromosome. We rationalized...
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 Bruce8-1e, nutcracker07259 or Bruce10-1e, nutcracker07259, 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 (Prosa6T – 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 nutcrackerms771, in which no ICs form, Alpha6T-GFP persisted in the nuclei (Fig. 6A, top left). By contrast, in the hypomorphic nutcracker07259, 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, nutcrackerms771 or nutcrackerms771 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 nutcrackerms771 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 (Prosa7 – FlyBase), Rpn3 and Rpt4 were unchanged in nutcrackerms771 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...
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 autoactivation 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; Schreadder 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

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.050088/-/DC1

References


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