RESEARCH COMMUNICATION

Tango7 directs cellular remodeling by the *Drosophila* apoptosome

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It is now well appreciated that the apoptosome, which governs caspase-dependent cell death, also drives non-apoptotic caspase activation to remodel cells. However, the determinants that specify whether the apoptosome acts to kill or remodel have yet to be identified. Here we report that *Tango7* collaborates with the *Drosophila* apoptosome to drive a caspase-dependent remodeling process needed to resolve individual sperm from a syncytium. In these cells, *Tango7* is required for caspase activity and localizes to the active apoptosome compartment via its C terminus. Furthermore, *Tango7* directly stimulates the activity of this complex in vitro. We propose that *Tango7* specifies the *Drosophila* apoptosome as an effector of cellular remodeling.

Supplemental material is available for this article.

Received April 5, 2013; revised version accepted June 21, 2013.

Apoptosis is the most common form of programmed cell death (PCD) and is intimately involved in development, tissue homeostasis, and disease (Fuchs and Steller 2011). Throughout the animal kingdom, execution of the intrinsic apoptotic pathway relies on the activation of the apoptosome. In *Drosophila*, this involves oligomerization of the initiator caspase pro-Dronc with the adaptor protein Dark to form the apoptosome complex (Ryoo and Baehrecke 2010). Apoptosome assembly converts the pro-Dronc zymogen into active Dronc, which subsequently activates executioner caspases Drice and Dcp-1. Upon dissociation from *Drosophila* inhibitor of apoptosis 1 (DIAP1), these caspases then cleave a specific subset of cellular substrates that, through a variety of mechanisms, leads to cell suicide and engulfment by macrophages (Hay and Guo 2006).

Paradoxically, apoptosome activity has recently been shown to drive diverse cellular processes without promoting cell death (Feinstein-Rotkopf and Arama 2009). Examples

of cellular remodeling such as enucleation of erythroblasts (Carlile et al. 2004), keratinocytes (Lippens et al. 2000) and lens fiber cells (Ishizaki et al. 1998); differentiation of macrophages (Sordet et al. 2002); formation of platelet cells from megakaryocytes (De Botton et al. 2002); dendritic pruning (Kuo et al. 2006; Williams et al. 2006); and, most recently, synaptic plasticity in the developing brain (Chen et al. 2012) have all been shown to require the apoptosome and/or caspase activity. An early precedent for caspase-dependent cellular remodeling came from studies of spermatid individualization in *Drosophila* (Arama et al. 2003, 2006; Huh et al. 2004). During this process, active caspases that can be visualized as syncytial spermatids are resolved into mature individual sperm through a specialized cytoskeletal structure known as the individualization complex (IC), which eliminates excess cytoplasmic content and encapsulates each sperm in plasma membrane (Fabrizio et al. 1998). In vivo, this process requires the apoptosome and caspase activity, since mutations in *Dark* or *Dronc* prevent normal caspase activation and coordinated IC movement, resulting in spermatogenesis defects and male sterility (Rodriguez et al. 1999; Huh et al. 2004; Arama et al. 2006).

These and other studies establish an essential role for the apoptosome, but whether this complex might be converted from a machine that demolishes cells to an engine that remodels them is not known. Furthermore, because the apoptosome is often sufficient for killing, prevailing models do not adequately explain how this complex functions without provoking apoptotic cell death. Through genetic and biochemical analysis, we found that *Tango7*, previously described by our group as an effector of cell death (Chew et al. 2009), collaborates with the apoptosome to remodel syncytial spermatid cysts during the individualization process. Collectively, our observations suggest that *Tango7* physically recruits the apoptosome to specify this complex for nonapoptotic remodeling of spermatids.

Results and Discussion

In order to characterize *Tango7* function in vivo, we accessed a community resource (Cooper et al. 2008) to screen a heavily mutagenized second chromosome collection for deleterious point mutations in the *Tango7* locus. Ten *Tango7* variants were recovered and placed in *trans* to deficiencies that delete the *Tango7* locus (see the Materials and Methods). One variant was lethal, another was viable but male-sterile, and the remaining variants had no obvious phenotypes. We confirmed the lethal variant to be nonsense mutation Q135* and the male-sterile variant to be nonsense mutation W358*, which we designated *Tango7^E* and *Tango7^L*, respectively. Complementation studies revealed that *Tango7^E* is a null allele and *Tango7^L* is a hypomorphic allele. Specifically, *Tango7^E* is lethal at the first instar larval stage when tested in *trans* to itself and *Df(2R)Exel7130*, a deficiency that uncovers *Tango7*. *Tango7^L*, however, is viable but male-sterile in all allelic combinations (Fig. 1B). Furthermore, *tango7^{L/L}* flies are healthier than *tango7^{L/E}* flies, which mostly die during eclosion or soon thereafter. To confirm that *Tango7^L* produces a truncated protein, we blotted lysates from wild-type, heterozygous mutant, or homozygous

[Keywords: apoptosome; cellular remodeling; caspase activity; apoptosis; cell death]

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Figure 1. *Tango7* is an essential gene, and its C terminus is required for male fertility. (A) *Tango7* encodes a 387-amino-acid protein with a conserved PCI domain. Two nonsense mutations were recovered by FLY-TLL (arrowheads above gene structure). Gray boxes are exons, and white boxes are untranslated regions. (B) *Tango7^L* is viable but male-sterile in all allelic combinations. *Tango7^E* is lethal in *trans* to itself and *Df(2R)Exel7130* and semiviable in *trans* to *Tango7^L*. Viable *tango7* mutants also exhibited a “wrinkled” wing phenotype characteristic of *W¹* mutants, a dominant *Hid* allele (Abbott and Lengyel 1991). A genomic fragment rescue, *Tango7^{VK006}*, rescued viability, male sterility, and the wrinkled wing phenotype of *tango7* mutants. (C) Lysates from Kc167 cultured cells, wild-type embryos, and testes of various genotypes were blotted with anti-Tango7 (see the Materials and Methods). Note that the mutant *Tango7^L* protein was stably expressed in both heterozygote and mutant testes in lanes 3 and 4, respectively. (Lane 5) One copy of the *Tango7^{VK006}* rescue (R) in the mutant background effectively expresses the full-length protein in the testes.

mutant tissue with a monoclonal *Tango7* antibody (Fig. 1C; for antibody, see the Materials and Methods). Samples from cultured Kc167 cells, wild-type embryos, and wild-type testes all showed a prominent band at the expected ~44-kD size (Fig. 1C). The premature stop in the *Tango7^E* allele eliminates the epitope recognized by anti-Tango7, but as seen in Figure 1C, heterozygous *Tango7^{L/+}* testes produced both the full-length protein and the predicted shorter variant at ~40 kD. Furthermore, the homozygous *tango7^{L/L}* testes produced only the shorter variant, as expected (Fig. 1C). Taken together, we conclude that *Tango7^E* is a null allele and *Tango7^L* is a hypomorphic allele expressing a truncated variant that lacks the C-terminal 30 amino acids.

Like *dark* and *dronc* mutants (Rodriguez et al. 1999; Chew et al. 2004; Huh et al. 2004; Xu et al. 2005; Arama et al. 2006), all viable *tango7* mutants were male-sterile and exhibited several wing phenotypes (Fig. 1B). A wrinkled wing phenotype characteristic of cell death mutants (Abbott and Lengyel 1991; Grether et al. 1995) appears at a modest frequency in *tango7^{L/L}* adults and at higher frequencies in *tango7^{L/E}* and *tango7^L/Df(2R)Exel7130* flies (Fig. 1B; Supplemental Fig. 1). We also noted a low penetrance of mild blemishing and upward curved wings (Supplemental Fig. 1), similar to mutants for *Dark* and *Dronc* (Chew et al. 2004; Xu et al. 2005).

Although viable *tango7* mutants exhibit wing phenotypes characteristic of apoptotic mutants (Abbott and Lengyel 1991; Chew et al. 2004; Xu et al. 2005), we did not observe global cell death defects in zygotic null embryos as examined by acridine orange staining (Abrams et al. 1993). It is possible that, like *dark* and *dronc* embryos, zygotic expression of *Tango7* might be dispensable for embryonic PCD because of maternally loaded transcript (Chew et al. 2004; Akdemir et al. 2006). In support of this,

we detected abundant *Tango7* transcript in *Df(2R)Exel7130* homozygous embryos that genetically lack the *Tango7* locus (Supplemental Fig. 2). Therefore, like other apoptosome mutants (Chew et al. 2004; Akdemir et al. 2006), zygotic sources of *Tango7* are not rate-limiting for most cell deaths in the embryo. To test for more subtle phenotypes, we stained for markers that detect supernumerary cells in known cell death-defective mutants (Rogulja-Ortmann et al. 2007). In *tango7^{L/E}* embryos produced by *tango7^{L/L}* females, extra dHb9⁺ neurons were detected, but persisting Kr⁺ cells were not seen (Supplemental Fig. 2C–F). Therefore, *Tango7^L* exhibits maternal effect PCD-associated phenotypes in some but not all contexts.

To determine whether the sterility of *tango7* males, like apoptosome mutants (Rodriguez et al. 1999; Huh et al. 2004; Arama et al. 2006), might be caused by defective caspase activation prior to spermatid individualization, we stained wild-type, *tango7^{L/L}*, and *tango7^{L/E}* testes with cleaved Caspase-3 antibody (anti-CC3), a marker of Dronc substrate cleavage (Fan and Bergmann 2010). Strikingly, *tango7* mutant testes completely

failed to stain for anti-CC3, indicating that Dronc fails to cleave its substrates in *tango7* mutant cysts (Fig. 2A). Furthermore, wild-type and *tango7* mutant spermatids were similarly positive for AXO49 (Fig. 2B), a marker for advanced spermatogenesis (Bressac et al. 1995). Therefore, the absence of anti-CC3 staining in *tango7* mutants reflects an authentic and specific failure in caspase activation rather than developmental arrest.

To examine whether failure in caspase activation was coupled to spermatid individualization defects, we stained wild-type and *tango7* mutant testes with phalloidin, which binds to the actin of the investment cones that make up the IC. In wild-type testes, ICs form at the nuclei of spermatids and then migrate synchronously through the syncytia toward the opposite end of the cyst, expelling cytoplasmic content and wrapping each sperm in plasma membrane (Fig. 2F). However, like animals compromised for *dronc* and *dark* (Huh et al. 2004), *tango7* mutant ICs do form properly but either stall at the nuclei or move asynchronously through the syncytia (Figs. 2C, 3F).

To definitively assign these phenotypes to *Tango7*, we generated a rescue strain by site-specific integration of a 20-kb bacterial artificial chromosome (BAC) spanning the *Tango7* locus into the fly genome, which we named *Tango7^{VK006}*. One copy of this genomic fragment expresses full-length *Tango7* in the testes at levels comparable with wild-type (Fig. 1C) and can effectively rescue sterility (Fig. 1B) as well as caspase activation and individualization defects in *tango7^{L/L}* and *tango7^{E/E}* males (Fig. 2D,E). Additionally, *Tango7^{VK006}* reversed the lethality of the *Tango7^E* allele, the semiviability of *tango7^{L/E}* adults, and all of the observed wing phenotypes (Fig. 1B). Taken together, these genetic data demonstrate that, like the apoptosome components *Dronc* and *Dark*, *Tango7* is

Figure 2. *tango7* mutants are defective for caspase activity and apoptosome-dependent cellular remodeling. [A–E] Confocal micrographs of immunofluorescence on whole-mount testes. (A) Testes stained with anti-CC3, a surrogate for Dronc substrate cleavage (Fan and Bergmann 2010). Spermatid cysts in wild-type testes are positive for anti-CC3, but >90% of *tango7* mutant cysts failed to stain, reflecting a defect in Dronc activity (~10% stained weakly for anti-CC3). (B) Testes stained with anti-AXO49, which binds polyglycated β 2 tubulin at the onset of individualization (Bressac et al. 1995). *tango7* mutant cysts are positive for anti-AXO49, indicating that spermatogenesis proceeds normally in these mutants. Signal here was pseudocolored cyan. (C) Testes stained with phalloidin, which binds to the actin in ICs (open arrowheads). Wild-type ICs form at the basal end of cysts and then migrate toward the distal end, whereas *tango7* mutant ICs form at the basal end but then either stall or move in an asynchronous fashion (see also Fig. 3F). (D,E) One copy of the *Tango7^{VK006}* rescue fragment restored fertility, caspase activation (anti-CC3), and individualization (phalloidin) in spermatid cysts of *tango7^{L/L}* (D) and *tango7^{E/E}* (E) testes. (F) Schematic of spermatid individualization in the testes. Each spermatid cyst (gray lines) contains 64 syncytial spermatids. Caspase activation occurs upon formation of the IC at spermatid nuclei and is most prominent at the cystic bulge, which forms as the IC translocates through the syncytia. The inset shows a more detailed view of the cystic bulge, where 64 syncytial spermatids (only four are shown for simplicity) are resolved into individual sperm by the IC and wrapped in their own plasma membrane (black lines). Arrows represent direction of individualization. (Red) caspase activation; (blue) nuclei; (yellow) IC. Bars, 150 μ m.

similarly required for nonapoptotic caspase activation and individualization in the *Drosophila* testes.

In spermatids that are being remodeled, the active apoptosome localizes to the IC at the nuclei and the cystic

bulge, as indicated by active Dronc staining (Huh et al. 2004). To determine whether Tango7 also localizes to this structure, we stained wild-type testes with anti-Tango7. Indeed, Tango7 conspicuously localized to the cystic bulge and the waste bag (Fig. 3A–E, white and yellow boxes). Furthermore, this protein notably colocalized with anti-CC3 at these structures but not at other regions (Fig. 3A–C’), suggesting that Tango7 plays a role in caspase activation specifically at the IC. We further observed that Tango7 discretely localized to the leading edge of wild-type investment cones in the cystic bulge (Fig. 3E, yellow arrow). In contrast, the truncated *Tango7^L* protein (which lacks C-terminal 30 amino acids) was notably absent from the leading edge of asynchronously moving investment cones in *tango7^{L/L}* spermatids (Fig. 3F, yellow arrow). These findings suggest that the extreme C terminus of Tango7 is required for its localization to the IC and thus its function in cellular remodeling.

Our *in vivo* observations suggest that Tango 7 genetically interacts with the apoptosome to regulate nonapoptotic caspase function. To test whether Tango7 might directly affect apoptosome activity, we reconstituted the apoptosome *in vitro* using recombinant Dronc and Dark in the presence or absence of recombinant Tango7 and assayed for caspase activity using catalytically dead pro-Drice^{C/A} as a substrate. As seen in Figure 4A, the presence of Tango7 stimulated cleavage of pro-Drice^{C/A} by the apoptosome in a dose-dependent manner (Fig. 4A). This enhancing effect was heat-sensitive (Fig. 4B, lane 3) and was not seen when a catalytically dead active site Dronc mutant (Meier et al. 2000) was used (Fig. 4B, lane 4). Furthermore, consistent with previous studies on the fly apoptosome (Yuan et al. 2011), cytochrome c was not similarly active in these assays (Supplemental Fig. 3C). To probe whether Tango7 interacts with the apoptosome directly, we tested for interactions between recombinant proteins *in vitro*. Tango7 did not interact with an irrelevant His-tagged protein (Supplemental Fig. 3G) but did bind to Dronc or Dark individually or together (Fig. 4C). Combined, these results show that Tango7 can directly stimulate apoptosome activity *in vitro*.

To test whether similar physical interactions occurred *in vivo*, we performed coimmunoprecipitation experiments in cultured S2R⁺ cells cotransfected with Tango7-V5 and either Flag-Dronc or Dark-Myc constructs. In these experiments, Tango7 interacted with Dronc (Supplemental Fig. 3A) and with Dark (Fig. 4E). Likewise, in re-

Figure 3. Tango7 localizes to the active apoptosome compartment via its C terminus in individualizing spermatids. Confocal micrographs of immunofluorescence on whole-mount testes. (A–C'') Wild-type testes stained with anti-Tango7 and anti-CC3, a marker of Dronc substrate cleavage (Fan and Bergmann 2010). (A–A'') Tango7 colocalizes with anti-CC3 in the cystic bulge (white box) and in waste bags (yellow box), which harbor active Dronc caspase (Huh et al. 2004). (B–B'') Higher magnification of the cystic bulge in A. (C–C'') Higher magnification of a waste bag from a different wild-type testis. (D–F) Testes stained with DAPI (blue), anti-Tango7 (green), and phalloidin (red). (D) In wild-type testes, Tango7 is associated with the IC in the cystic bulge (white box) and the waste bag (yellow box). (E) Higher magnification of the cystic bulge in D. (Inset) Within the cystic bulge, Tango7 is discretely localized to the upper edge of investment cones (yellow arrow). (F) In *tango7^{L/L}* testes, the C-terminal truncated Tango7^L protein failed to localize to the upper edge of investment cones (inset, yellow arrow), and ICs move asynchronously (white arrows). Bars: A–A'', D, 150 μ m; B–C'', 50 μ m; E, F, 25 μ m. Curved arrows represent direction of individualization.

ciprocal immunoprecipitation experiments, these same interactions were detected (Fig. 4D; Supplemental Fig. 3B). In these assays, we also observed that Dronc and Dark interacted with a modified form of Tango7 (Fig. 4D; Supplemental Fig. 3B). Taken together, these results demonstrate that Tango7 interacts with the apoptosome components Dark and Dronc in vitro and in vivo.

Like animals mutated for apoptosome genes, we show here that viable *Tango7* males are sterile because they fail to initiate a caspase-dependent process that remodels syncytial cysts to produce individualized sperm. These charac-

teristic defects established a classic precedent for how apoptosome activation does not inevitably drive cell killing but can instead be repurposed for nonapoptotic processes during spermatogenesis (Arama et al. 2003; Huh et al. 2004; Feinstein-Rotkopf and Arama 2009). How might *Tango7* collaborate with the apoptosome as spermatids are remodeled? Since Tango7 was originally described in a collection of targets linked to Golgi organization (Bard et al. 2006), we examined a Golgi marker in *tango7^{L/L}* tissue but found no indication that this organelle was affected (Supplemental Fig. 4). Studies of Tango7 orthologs in *Caenorhabditis elegans* (Luke-Glaser et al. 2007) and in yeast (Zhou et al. 2005) suggest that the protein could also act as a noncore factor associated with the COP9 signalosome complex and/or the eIF3 translation complex. These modalities could certainly be relevant in vivo, but the in vitro activities seen here with recombinant Tango7 (Fig. 4A,B) suggest functions independent from these multiprotein complexes. Furthermore, since *tango7^{L/L}* mutants are fully viable, it seems doubtful that systemic defects in either of these complexes can account for caspase-dependent spermatogenesis defects seen in these animals. Instead, we propose that *Tango7* directly engages the apoptosome to specify this holoenzyme for cellular remodeling. Several pivotal observations support this. First, the action of Tango7 is clearly required for apoptosome-dependent caspase activity and remodeling of spermatids. Second, Tango7 localizes to the active holoenzyme compartment in vivo and physically binds apoptosome proteins in vitro and in cultured cells. Third, Tango7 is one of few proteins (and the first of its kind in *Drosophila*) able to stimulate apoptosome activity in vitro. Together these observations suggest that Tango7 functions as a direct regulatory component of this complex, acting also perhaps as a scaffold or chaperone that could promote remodeling by recruiting the active apoptosome to the IC. Consistent with this, Tango7^L (which truncates 30 amino acids from the C terminus) fails to localize to the IC in caspase-defective *tango7^{L/L}* testes. Furthermore, like active Dronc, Tango7 localized to the investment cones of migrating ICs and correlated with active caspases at this structure but not with anti-CC3 staining elsewhere in the testes.

To further explore this scenario, we sought to localize active Dronc in *tango7* spermatids, but unfortunately, this particular antisera is no longer available. Nevertheless, combined with our genetic observations, these data establish that the C terminus of Tango7 is dispensable for viability but required for apoptosome-dependent remodeling and localization to the IC. Hence, *Tango7^L* is an allele-specific variant that uncouples essential functions from nonessential functions. Consistent with this, *Tango7^L* clones grew normally, but *Tango7^E* clones arrested after several cell divisions (Supplemental Fig. 5).

Figure 4. Tango7 interacts with components of the apoptosome and stimulates apoptosome activity. (A,B) Apoptosome activity assays detecting cleavage of pro-Drice substrate by the apoptosome. In A and B, filled arrowheads denote substrate (pro-Drice^{C/A}-6xHis), and open arrowheads indicate product (cleaved Drice^{C/A}-6xHis). (A) Tango7 stimulates cleavage of Drice by the apoptosome in a dose-dependent manner. The top blot in A is a lighter exposure of the pro-Drice^{C/A} band, indicated by an asterisk. (B) This stimulation effect by Tango7 was heat-sensitive (lane 3), and the appearance of product required wild-type Dronc (lanes 4,5). H in lane 3 indicates that Tango7 was heat-inactivated before it was added to the reaction. M in lanes 4 and 5 indicates that recombinant active site mutant Dronc, Dronc^{C/A}, was used. (C) Tango7 directly interacts with Dark and Dronc in vitro. Recombinant 6xHis-Dark-6xHis, Dronc-6xHis, and Tango7-V5-6xHis were incubated together in the combinations indicated. Complexes were immunoprecipitated using anti-V5 and detected using anti-His. This interaction is specific, as Tango7 does not pull down an irrelevant protein control (Supplemental Fig. 3C). An asterisk indicates a cross-reacting background band. (D,E) Tango7 physically interacts with Dark and Dronc in cultured cells. (D) Flag-Dronc interacted with Tango7-V5 in cultured cells. Notice that Flag-Dronc also bound a post-translationally modified form of Tango7, Tango7-V5*. This modified form of Tango7 was not affected by phosphatase treatment (data not shown). (E) Tango7-V5 interacted with Dark-Myc in cultured cells. Input controls for A–C and reciprocal immunoprecipitations for D and E can be found in Supplemental Figure 3.

Mechanisms that recruit the apoptosome to a subcellular structure, together with restraints imposed by the ubiquitin–proteasome system (Kuo et al. 2006; Arama et al. 2007; Kaplan et al. 2010), could limit caspase activity within discrete subcellular compartments. This scenario explains how partial demolition of subcellular structures might occur, but conceivably, the apoptosome could also exert constructive roles during remodeling as well. Therefore, it will be interesting to investigate whether properties of the apoptosome (e.g., substrate specificity) are affected by Tango7. Likewise, it would also be interesting to test whether Tango7 is generally needed to remodel cells in other tissues (Geisbrecht and Montell 2004; Kuo et al. 2006) or whether Tango7 counterparts might be similarly required in other species. Viewed from this perspective, the *Tango7^L* truncation allele opens unique opportunities for investigating how the apoptosome is diverted from cell suicide functions toward cell remodeling activity.

Materials and methods

Fly strains and husbandry

CantonS flies were used as wild-type controls. *Tango7^L* and *Tango7^E* alleles were recovered by the Seattle *Drosophila* TILLING Project (Cooper et al. 2008), which screened for point mutations in the second chromosome *CantonS*-derived Hawley lines. Variants were placed in *trans* to *Df(2R)Exel7130* and *Df(2R)50C-38*, which uncover the *Tango7* locus.

Immunofluorescence

Whole-mount testes were dissected in PBS, fixed, and stained as described in Arama et al. (2003), except that PBT (1× PBS, 0.1% Tween) was used for washes, and PBTA (1× PBS, 0.1% Tween, 1.5% BSA) was used for blocking and incubations with antibodies. The following antibodies and dilutions were used: rabbit anti-CC3 (Cell Signaling) at 1:500, mouse anti-AXO49 (Bressac et al. 1995) at 1:20, rhodamine-phalloidin (Invitrogen Molecular Probes) at 1:40, mouse anti-Tango7 (1N13) at 1:1000, and rabbit anti-GM130 at 1:500 (Abcam). Alexa-488 and Alexa-568 secondary antibodies (Invitrogen Molecular Probes) were used at 1:250. A custom monoclonal antibody (1N13) against Tango7 was raised at Abmart Antibody Company against epitope ELLGTYTADN. Micrographs were taken with Leica TCS SP5 and Zeiss LSM780 laser confocal microscopes.

Caspase activity assay

Activity assays were performed as in Yuan et al. (2011). Recombinant 6xHis-Dark-6His, Dronc-6His, and Tango7-V5-6His were mixed on ice, incubated for 30 min at 37°C, and then incubated overnight at 25°C to form the apoptosome complex. The next day, recombinant pro-Drice^{C/A}-6His was added and incubated for 6 h at 25°C. Subsequently, the proteins were resolved by SDS-PAGE and blotted with anti-His-HRP antibody (Invitrogen).

Immunoprecipitation

For in vitro immunoprecipitations, recombinant 6His-Dark-6His, Dronc-6His, and Tango7-V5-6His were mixed on ice, incubated for 30 min at 37°C, and then incubated overnight at 25°C to form the apoptosome complex. The proteins were diluted in buffer A (20 mM HEPES-KOH at pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT) and immunoprecipitated using anti-V5 antibody (Bethyl Laboratories) and protein G Dynabeads. For immunoprecipitations from cells, equal amounts of cell lysates were precleared with 40 μL of protein A/G Sepharose (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Lysates were then incubated overnight at 4°C with 2 μg of mouse anti-V5 (Bethyl Laboratories), rabbit anti-Myc (Invitrogen), or rabbit anti-Flag (Sigma). The following day, 40 μL of protein A/G Sepharose was added to the lysates, and the complexes were allowed to bind to the beads for 2 h at 4°C. Immunoprecipitates were then washed three times with 1 mL of ice-cold Triton lysis buffer, and the bound complexes were eluted with SDS sample buffer.

Acknowledgments

We thank Xiaoquin Tu, Nichole Link, and Leslie Durham for help with experiments and reagents. We are grateful to Marie-Hélène Bré for the AXO49 antibody, Joachim Seemann for the GM130 antibody, and Peter Michael for the NV18-6His recombinant protein. We thank Angela Diehl for the individualization schematic. We thank the rest of the Abrams laboratory for their support and expertise, and Dr. Michael Buszczak and Dr. Robin Hiesinger for their advice and comments. This work was supported by grants to J.M.A. from the National Institute of General Medical Sciences (R01GM072124), Cancer Prevention Research Institute of Texas (RP110076), the Ellison Foundation (AG-SS-2743-11), and the Welch Foundation (grant no. I-1727). This work was performed in laboratories constructed with support from NIH grant C06 RR30414.

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Genes Dev. 2013, **27**:

Access the most recent version at doi:[10.1101/gad.219287.113](https://doi.org/10.1101/gad.219287.113)

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