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B56-Associated Protein Phosphatase 2A Is Required For Survival and Protects from Apoptosis in *Drosophila melanogaster*

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Protein phosphorylation and specific protein kinases can initiate signal transduction pathways leading to programmed cell death. The specific protein phosphatases regulating apoptosis have been more elusive. Using double-stranded RNA-mediated interference (RNAi), the role of protein phosphatase 2A (PP2A) in cellular signaling was investigated. Knockdown of A or C subunits individually or of combined B subunits led to concurrent loss of nontargeted PP2A subunits, suggesting that PP2A is an obligate heterotrimer in vivo. Global knockdown of PP2A activity or specific loss of redundant B56 regulatory subunits caused cell death with the morphological and biochemical changes characteristic of apoptosis in cultured S2 cells. B56:PP2A-regulated apoptosis required caspases and the upstream regulators *dark*, *reaper*, *head involution defective*, and *dp53*. In *Drosophila* embryos, knockdown of B56-regulated PP2A activity resulted in apoptosis and failure of gastrulation, an effect that was blocked by concurrent RNAi of the caspase *Drice*. B56-regulated PP2A activity appears to be required upstream of dp53 to maintain a critical proapoptotic substrate in a dephosphorylated, inactive state, thereby preventing apoptosis in *Drosophila* S2 cells.

Precise regulation of apoptosis is an essential component of both development and tissue homeostasis. Dysregulation of apoptosis underlies diseases including cancer, neurodegenerative disorders, autoimmunity, and virus infections. A substantial amount is now known about the downstream effectors of apoptosis, including the regulators of caspase protease activity (recently reviewed in references 18, 22, and 41). Much of the cell death machinery, including initiator and effector caspases and their regulators, is present in the cell at all times but is maintained in an inactive state. Cell death can then be activated by a large variety of tissue-specific stimuli, including activation of developmental programs and extracellular hormone signals (or the lack thereof) and detection of intracellular damage. Damage-responsive proteins such as p53 signal to downstream pathways, resulting in cell cycle arrest or apoptosis. One widely used mechanism to prevent apoptosis is the use of phosphorylation-mediated survival signals, regulated in part by binding of extracellular ligands and cell adhesion to substrates. Survival signals that prevent activation of apoptosis come in part from protein kinases, including Akt/protein kinase B, mitogen-activated protein (MAP) kinase cascades, and IκB kinases that activate NF-κB. Conversely, proapoptotic signals can be generated following events such as DNA damage by activation of protein serine/threonine kinases, including ATM and Chk1. In several cases, the physiologic substrates of these proapoptotic kinases, regulatory proteins such as p53, are known.

While a series of protein kinases have been implicated in cell survival and programmed cell death, the counterregulatory protein phosphatases that must also control the balance of protein phosphorylation and hence the regulation of apoptosis are less well understood. In one example in *Drosophila melanogaster*, loss of the protein phosphatase 7 (PP7)-related RdgC protein phosphatase leads to hyperphosphorylation of rhodopsin and apoptosis specifically of photoreceptor cells (6, 23). Serine/threonine phosphatases may in general play an antiapoptotic role, since cells exposed to high concentrations of phosphatase inhibitors such as okadaic acid show extensive apoptosis (e.g., see reference 14). However, since okadaic acid can inhibit a number of intracellular protein serine/threonine phosphatases, including PP1, PP2A, PP4, and PP5, the specific phosphatase(s) required for cell survival remains unclear.

PP2A is an abundant heterotrimeric cellular serine/threonine-specific phosphatase that regulates a large number of cellular events, including DNA replication, Wnt signaling, tumorigenesis, and cytoskeleton functions (recently reviewed in references 19 and 43). The PP2A holoenzyme is a heterotrimer, consisting of a structural A subunit, a catalytic C subunit, and a variable targeting-regulatory B subunit (Fig. 1). The diverse functions of PP2A are mediated in large part by a diverse number of targeting B subunits that direct the phosphatase to distinct substrates and intracellular locations (7, 27, 32). There are three distinct B subunit families, designated B/B55/PR55, B′/B56/PR61, and B″/PR72. Each mammalian B subunit family is encoded by multiple genes, with multiple splice variants, generating an extraordinary diversity of these regulatory subunits. While a requirement for PP2A in cell survival is suggested by okadaic acid studies, in many instances, PP2A removes phosphate groups placed by prosurvival kinases. For example, the proapoptotic activity of BAD is suppressed by phosphorylation and is activated by dephosphorylation by PP2A, while the antiapoptotic activity of Bcl-2 can be activated by phosphorylation and suppressed by PP2A (9, 12).

In *Drosophila*, apoptosis during embryonic development is controlled by the expression of three tightly linked genes, *reaper* (*rpr*), *grim*, and *head involution defective* (*hid*), which

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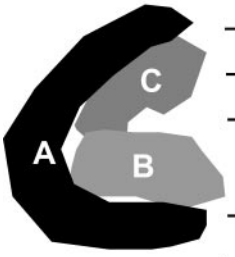

genes	*Drosophila*		mammal
A	dPP2A A	(1)	(2)
C	dPP2A C	(1)	(2)
B	dPR55	(1)	(4)
B'	dB56-1 dB56-2	(2)	(5)
B"	dPR72	(1)	(3)

FIG. 1. PP2A structure and genes in *Drosophila* and mammals. The structure of the PP2A holoenzyme is illustrated. Homologues of PP2A in *Drosophila* were identified by searching Flybase, the *Drosophila* genome, published literature, and the *Drosophila* expressed-sequence-tag database. The number of isoforms is indicated by the number in parentheses. Each structural A and catalytic C subunit in *Drosophila* is encoded by a single gene. As in mammals, there are three families of *Drosophila* B subunit genes; B/PR55 and PR72 are encoded by single genes, while B'/B56 is encoded by two distinct genes.

appear to integrate upstream signals regulating apoptosis (reviewed in references 1 and 40). *Drosophila* with a chromosomal deficiency removing these three genes is defective in apoptosis. *rpr*, *grim*, and *hid* are regulated by upstream signaling, and the expression of these genes, either individually or in combination, precedes cell death in diverse tissues. While no direct homologues have been identified in mammals, expression of *rpr*, *grim*, or *hid* in vertebrate systems causes apoptosis, suggesting a conservation of function (10, 13, 16, 31). Activation of the Ras/MAP kinase pathway leads both to down-regulation of *hid* expression and inhibition of Hid protein function by direct phosphorylation (3, 26). *rpr* expression is up-regulated by DNA damage, an effect that may be mediated by *Drosophila* p53 (dp53) (5, 20, 34). Cell death induced by *rpr*, *grim*, and *hid* requires caspase activation, but the mechanisms linking these upstream death activators to the downstream caspases are not completely clear. One mechanism involves interaction of these activators with inhibitors of apoptosis in *Drosophila* (dIAPs). On the other hand, the cell death induced by *rpr*, *grim*, and *hid* can be partially suppressed in flies mutant in *dark*, the *Drosophila* homologue of CED-4/Apaf1, suggesting that caspase activation requires Dark as well (35, 36).

Double-stranded RNA (dsRNA)-mediated interference (RNAi) with gene expression can produce a loss-of-function phenotype and has had wide application in the study of invertebrate development (reviewed in reference 38). The recent extension of this method to *Drosophila* cells in culture allows a further detailed analysis of specific signal transduction cascades (11). We took advantage of the smaller number of PP2A genes present in *Drosophila* to study the role of the phosphatase-targeting subunits in signal transduction in these cells. We find that loss of PP2A A or C or of all B subunits destabilizes the remaining subunits, suggesting that PP2A is stable only as a heterotrimer. Notably, RNAi-mediated knockdown of PP2A activity and, more specifically, loss of B56-targeted PP2A activity led to apoptosis characterized by both morphological changes and caspase activation. The cell death was prevented by RNAi of downstream apoptosis effectors, including *dark* and the caspases *drice*, *dredd*, and *dronc*. Similar findings were observed in *Drosophila* embryos, where combined RNAi of the two B56 genes led to widespread apoptosis and abortion of development prior to the extended-germ-band stage. These defects in embryogenesis are due solely to the induction of apoptosis, since they can be prevented by RNAi of *drice*. Epistasis analysis places B56-containing PP2A heterotrimers upstream of *Drosophila* p53, as well as of the regulators *rpr* and *hid* but not *grim*. Taken together, the data suggest that B56-containing PP2A heterotrimers promote *Drosophila* S2 cell survival by dephosphorylation of a critical substrate upstream of dp53 and that loss of activity of specific isoforms of PP2A can therefore regulate cell death.

MATERIALS AND METHODS

Cell culture. *Drosophila* Schneider S2 cells were cultured in 1× Schneider's *Drosophila* media (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 50 U of penicillin/ml and 50 μg of streptomycin/ml at 24°C.

dsRNA production. dsRNA was generated essentially as previously described (11) following protocols published on the Web (http://dixonlab.biochem.med.umich.edu/protocols/RNAiExperiments.html). Individual DNA fragments ranging from 700 to 850 bp in length, containing coding sequences for the proteins to be knocked down, were PCR amplified from a 0- to 4-h *Drosophila* cDNA library. Each PCR primer incorporated a 5′ T7 RNA polymerase binding site followed by sequences specific for the targeted genes (Table 1). The PCR products were purified using the PCR purification kit (Qiagen). dsRNA was synthesized from 1 μg of the purified PCR template by incubating a 20-μl reaction at 37°C for 12 to 16 h using a MEGASCRIPT T7 transcription kit (Ambion). The RNA products were then ethanol precipitated and resuspended in diethyl pyrocarbonate-treated water. dsRNA was annealed by incubation at 65°C for 30 min followed by slow (4 h to overnight) cooling to ambient temperature. The integrity of the dsRNA was examined by 1% agarose gel electrophoresis to ensure that the majority of the dsRNA ran as a single band of the predicted size. dsRNA was stored as aliquots at −80°C until use.

RNAi in *Drosophila* S2 cell cultures. RNAi was applied to S2 cells as described earlier (11). Briefly, S2 cells were harvested by centrifugation at 200 × *g* for 10 min and resuspended in *Drosophila* expression system serum-free medium (Invitrogen). One milliliter at 10^6 cells/ml was plated per well of a six-well cell culture dish. Fifteen to 20 μg of dsRNA per message was added to the culture media. After mixing, the cells were incubated for 1 h at 24°C followed by addition of 2 ml of 1× Schneider's media containing 15% heat-inactivated fetal bovine serum. The cells were cultured for 3 or 4 days to allow for turnover of the target protein. For concurrent RNAi (co-RNAi), 15 to 20 μg of each dsRNA was added to the S2 cells.

RNAi in *Drosophila* embryos. dsRNAs were diluted to 2.5 μM in injection buffer (2 mM EDTA, 80 mM KCl, 40 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], pH 8.0) and were injected into syncytial blastoderm stage embryos 45 to 90 min after egg laying as described previously (45). Following injection, embryos developed under a layer of halocarbon oil for 6 to 8 h and were then stained with acridine orange as previously described (2). Embryos were visualized using a Bio-Rad MRC-1024 confocal laser scanning microscope. Images were generated by overlaying *z* series sections.

Immunoblot analysis. Cells were harvested by centrifugation at 200 × *g* rpm for 10 min, and the pellets were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, protease cocktail [Roche], and 0.1% NP-40). The resulting samples were homogenized on ice using a rotary pestle followed by centrifugation at 12,000 × *g* for 15 min at 4°C. The supernatants (approximately 100 μg of protein) were resolved on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Immunoblots were performed as previously described (28). The antibodies used were 109-3AP (immunopurified rabbit anti-human PP2A C, which recognizes *Drosophila* PP2A C) (33); rat anti-*Drosophila* PP2A A (39); rat anti-*Drosophila* PR55/Twins (the generous gift of S. Yanagawa, Kyoto University, Kyoto, Japan); M787, a rabbit anti-human B56 that recognizes dB56-1 (the generous gift of Marc Mumby, University of Texas Southwestern Medical Center) (42); and UT-31 (rabbit anti-human casein kinase I ε CKIε, which recognizes an epitope present in the *Drosophila* CKIε homologue, Dbt). Protein abundance was quantitated by densitometry and NIH Image software.

RT-PCR. Total RNA was isolated from S2 cells with Trizol reagent following the manufacturer's instructions (GIBCO). The targets to be amplified were

TABLE 1. Genes and RNAi primers used in this study

Gene	Accession no.	Primers used for making dsRNA[a]
dPP2A A	M86442	F: GACTTCTGCGCCAATCTGGAC R: ATCAATGACGCTGGCCTCCAG
dPP2A C	X55199	F: TGCGGAGATGTCCACGGTCAG R: GAAATAATCGGGTGTTCTTCG
dB56-1	AJ277140	F: TCTTCCAACCCGAATGGTGCC R: GCGACATAATGTACTCGTTGTTC
dB56-2	AF145696	F: GAGGACGATCCGACACTGGAG R: CATGATCGGCATGATGACCGC
dPR55	D13004	F: GTTAATTCGGATCAGGAGACC R: TATGAAGAGGTTATTGGTCGC
dPR72	CG4733	F: AGATTCCGTTCGTACATCGTG R: GAAGGTATCGAAGAAGACGTG
Dbt	AF055583	F: TCGCTGAAGCACATTCCCTATC R: CATCCGTATCGAGGGCCGACG
dAXIN	AF086811	F: AGACTGGAAATCCGACCTCCTG R: AATGCCACTGTCTGTGTTCATG
Dronc	AF104357	F: CAATCACGTTTCAACCGAGGC R: GCCAAGATTATCATAGGCACC
Drice	Y12261	F: AACATGCGCCACAAGAACCGC R: GTCGCTGAAACGCAGAATGCG
Dredd	AF007016	F: CCACTGCGTAGACGGGATGGC R: TGGAAAGTACACGTGCTGGCG
dIAP-1	L49440	F: GAGACGCGATTAAAGACCTTCAC R: ACCCTTCATCAGCTTGACGAATC
dapaf1L dapaf1S	AB027531 AB027532	F: AGCTTCGAATCCACAGCATTCAG R: GTCGTCGGAATCGAATGTTTTTG
dp53	AF244918	F: TCTCTTTGGATGTACTCGATTCCG R: TTCCTTATTGGGCGACGTAATAGC
Reaper	L31631	F: GAACGAACTCGAAAATACGAAAG R: ACTCATCTTCGTTTTTTTCTGGGG
Hid	U31226	F: GAGTACAACTTCTTCCGGCAGCAG R: AACGAAAACGGTCACAACAGTTGG
Grim	U61976	F: TACAAGACTTAAAGTGCAAGCAGT R: AAACTCGTTCCTCCTCATGTGTCC

[a] T7 promoter sequence GAATTAATACGATCTACTATAGGGAGA precedes both forward and reverse primers.

approximately 300 bp in length, with at least one of the primers lying outside the region targeted by dsRNA. Target messages were reverse transcribed and amplified using the Titan One Tube reverse transcriptase PCR (RT-PCR) system (Roche).

Determination of caspase activity, DAPI staining, and viability assays. The caspase assay was performed with the ApoAlert Caspase-3 Colorimetric Assay Kit following the manufacturer's instructions (Clontech). Briefly, 50 μl of cell extracts made from 3×10^6 S2 cells was mixed with 50 μl of 2× reaction buffer and 5 μl of the colorimetric peptide substrate DEVD-*p*-nitroanilide. After incubation at 37°C for 1 h, the sample was centrifuged at 12,000 × *g* for 5 min, and the optical density at 405 nm was determined. For 4′,6′-diamidino-2-phenylindole (DAPI) staining, S2 cells were grown on coverslips precoated with polylysine. Four days after the addition of dsRNA, coverslips were washed once with phosphate-buffered saline (PBS) (pH 7.4), fixed with a mixture of methanol and acetone (1:1) for 10 min, washed once with PBS, and stained with 1 μg of DAPI/ml in PBS for 10 min at room temperature. Coverslips were then washed once with PBS and observed under UV light. Cell viability was examined by staining cells with trypan blue.

RESULTS

RNAi suggests that dPP2A is an obligate heterotrimer. In order to investigate PP2A function in *Drosophila* S2 cells, genes encoding PP2A subunits (denoted dPP2A) were identified by searching Flybase and published reports and by performing BLAST searches of the *Drosophila* genome and expressed-sequence-tag databases (Fig. 1). Previous studies have characterized *Drosophila* PP2A A, C, and B/PR55 subunits, each of which is encoded by a single gene. While PR55 has two splice variants, the transcripts vary only in the 5′ untranslated region and the extreme amino terminus (30). Additionally, a single B'/B56 homologue encoded by a locus on 90E3-6 was recently identified that interacts with and targets PP2A activity to the Hox protein SCR (Sex-combs reduced) (4). Database searching identified a second B56 cDNA, denoted here as B56-2 and encoded by the gene *LD34343*/*CG5643* mapping to 98A3 on *Drosophila* chromosome III. B56-2 is 68% identical to *Drosophila* B56-1 and is most closely related to human B56ε and -α (32, 33). An additional predicted gene (*CG7913*), from the same locus as B56-1, is described in Flybase; however, its poor alignment with bona fide B56 subunits suggests that *CG7913* has been incorrectly deduced from the genomic sequence. This sequence was not studied further. A single PR72-related gene, *CG4733*, maps cytologically to 92B8-9. It encodes a predicted protein of 888 amino acids 67% similar and 54% identical to human PR72 over a 407-amino-acid region. *Drosophila* PR72 is also closely related to human PR48, a B" subunit implicated in the regulation of the initiation of DNA replication (49). Thus, in contrast to the mammalian genome in which each PP2A subunit is encoded by multiple genes, in *Drosophila*, each subunit except B56 is encoded by a single gene.

To assess the function of these *Drosophila* PP2A subunits, we utilized RNAi in Schneider S2 cells in culture. RNAi, a powerful method for dissecting signal transduction pathways, is incompletely understood but inhibits cognate protein expression with near-total loss of targeted mRNA (38). RNAi can be performed in insect cell cultures by simply adding dsRNA to the culture medium (11). As an initial test of the efficacy of the RNAi in down-regulation of PP2A subunit expression, S2 cells were exposed to a 750-bp dsRNA derived from dPP2A A. After 4 days, the amount of A subunit protein remaining was examined by immunoblot analysis. RNAi of dPP2A A resulted in a >95% reduction of the A subunit protein (Fig. 2A, top panel, lane 2), indicating effective interruption of A subunit synthesis. RNAi was specific, since parallel mock treatment had no effect on A subunit protein (Fig. 2A, lane 3), and RNAi of *dAxin* dramatically reduced *dAxin* mRNA (data not shown) without altering A subunit protein (lane 1).

The A subunit of PP2A serves as a structural scaffold that binds both the C and B subunits (Fig. 1). To determine if loss of the A subunit leads to decrease of other subunits within the dPP2A heterotrimer, B and C subunit protein abundance was assessed after RNAi of the A subunit. As shown in lane 2 of

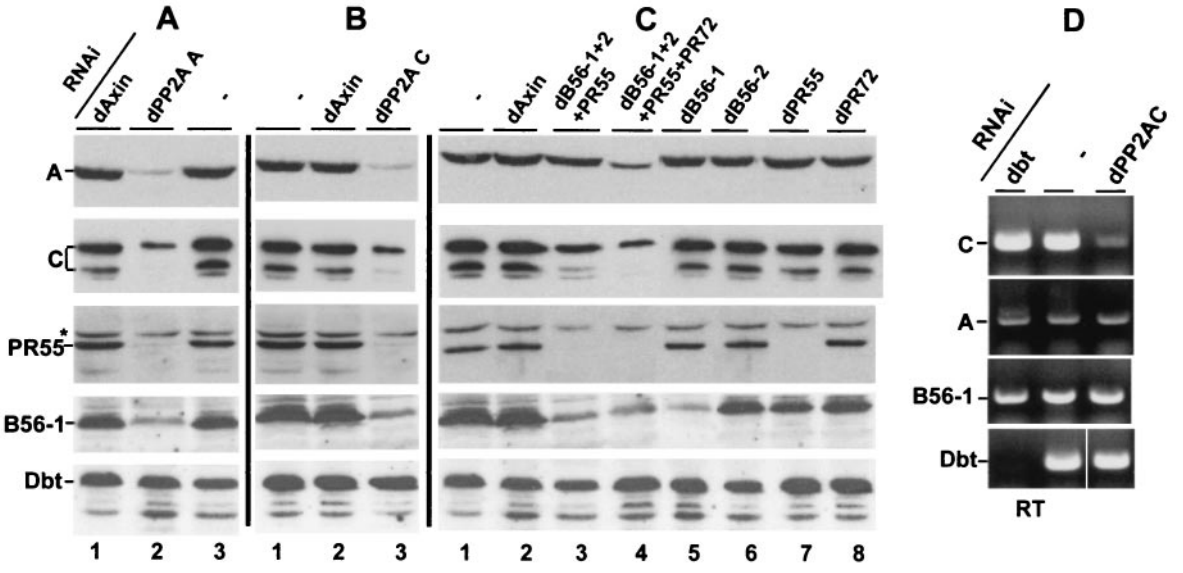

FIG. 2. RNAi-mediated knockdown suggests that PP2A is an obligate heterotrimer in S2 cells. Fifteen micrograms of each indicated dsRNA was added to S2 cells in six-well dishes as described (Materials and Methods), and the cognate proteins were examined by immunoblot analysis 96 h later. Dbt, the *Drosophila* CKIε homologue, was examined both as a control for equal protein loading and as a test of the specificity of RNAi. The dsRNA added is indicated along the top. −, no dsRNA added. The star indicates a cross-reacting band detected by the antibody directed against PR55. (A) RNAi of dPP2A A down-regulates A, C, and B subunits. (B) RNAi of dPP2A C down-regulates A, C, and B subunits. (C) RNAi of the four known B subunits in *Drosophila* down-regulates A and C subunits. (D) RNAi of dPP2A C has no effect on the mRNA levels of A and B subunits. Total RNA was extracted from S2 cells 4 days following RNAi of the indicated genes, followed by RT-PCR (RT) for A, C, B56-1, and Dbt. PCR products were separated by electrophoresis on 1% agarose and examined by ethidium bromide staining.

Fig. 2A, RNAi of dPP2A A not only mediated loss of its own protein but also down-regulated the soluble C, PR55, and B56-1 subunits. Loss of the B and C subunits was specific, since the unrelated protein Dbt was not affected by the A subunit RNAi and since RNAi of dAxin had no effect on PR55, B56-1, and C subunit protein. The data suggest that the majority of B and C subunits in the cell are present in an ABC heterotrimer and are unstable or insoluble in the absence of the A subunit.

RNAi of dPP2A C resulted in a marked reduction in C subunit protein and similarly led to a reduction in A and B subunit protein abundance (Fig. 2B, lane 3). To differentiate between decreased transcription versus destabilization of the remaining PP2A subunits, mRNA levels of A, B, and C subunits were examined by RT-PCR after C subunit RNAi (Fig. 2D). While RNAi of the C subunit led to specific loss of C mRNA, A and B56-1 mRNA levels were constant (Fig. 2D, lane dPP2A C), despite the loss of their protein product. Hence, similar to results seen with the A subunit, the C subunit also appears essential for stable PP2A protein. We note that these data do not exclude the possibility that loss of C subunit mRNA causes a decrease in translation of A and B subunit mRNA.

Combined RNAi of all identified B subunits suggests that B subunits are similarly required for stable A and C subunits. RNAi of B56-1 and PR55 individually produced the expected decrease in cognate protein levels (Fig. 2C, lanes 5 and 7). This effect was specific, with no effect on A or C protein abundance. Combined RNAi of B56-1, B56-2, and PR55 led to an approximately 30% decrease in A and C subunit protein abundance (Fig. 2C, lane 3). When all four known B subunits were subject to RNAi, there was an 80 to 90% decrease in both A and C subunit protein abundance (Fig. 2C, lane 4). This effect was specific to the PP2A subunits, as there was no change in the unrelated Dbt protein. While PR72 protein abundance was not assessed, the finding that triple knockdown of B56-1, B56-2, and PR55 had only a modest effect on A and C protein, while the quadruple RNAi (adding PR72 dsRNA) markedly diminished A and C protein abundance, suggests that PR72 is present and functional in the PP2A heterotrimer in S2 cells (comparing lanes 3 and 4 in Fig. 2C). One concern with adding dsRNA-targeting multiple distinct genes in the same reaction is that the cellular RNAi machinery could be saturated, leading to decreased efficiency of mRNA degradation. However, we were able to target four genes (B56-1, B56-2, PR55, and PR72) with no apparent decrease in efficiency (Fig. 2C, lane 4).

Taken together, these data strongly suggest that the majority of dPP2A present in S2 cells is an ABC heterotrimer and that the B subunits together contribute substantially to the stability of PP2A in vivo. Furthermore, these data support the conclusion that the major *Drosophila* A, B, and C subunit genes expressed in S2 cells have been identified. As RNAi of one gene (C subunit) did not influence the mRNA levels of other PP2A genes (Fig. 2D and data not shown), the down-regulation of the A and B PP2A subunits is posttranscriptional. Considering that the formation of the PP2A heterotrimer is dependent on the presence of each subunit, it seems likely that this down-regulation is due to acceleration of protein degradation, although effects on mRNA translation and protein solubility have not yet been ruled out. These findings support the hypothesis that free PP2A subunits (including AC heterodimers) unable to form a heterotrimer in vivo are inherently unstable and are rapidly degraded.

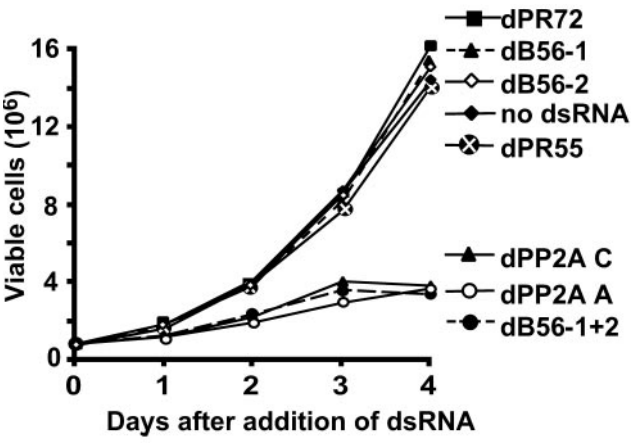

FIG. 3. PP2A and its B56 regulatory subunits are required for S2 cell multiplication. Viable S2 cells per well (determined by trypan blue exclusion) were counted at days 1 to 4 after the addition of the indicated dsRNAs. The B56 subunits appear to be redundant for cell multiplication.

RNAi of PP2A and its B56 regulatory subunits decreases viability of S2 cells. After the specificity and efficiency of RNAi of dPP2A were established, RNAi was utilized to examine the roles of PP2A in cell proliferation. We noted that, when RNAi of the A or C subunit was performed, the number of viable cells increased only slightly over time in comparison with that of the mock-treated S2 cells (Fig. 3). Since RNAi of the A subunit also down-regulated C subunit protein (Fig. 2A), the diminished cell accumulation caused by RNAi of A and C subunits is likely to be due to loss of PP2A catalytic activity. To test whether this decrease in viability was due to loss of a specific PP2A heterotrimer or to global loss of PP2A catalytic activity, individual B subunits were removed by RNAi. RNAi of the dPR72, dPR55, dB56-1, or dB56-2 subunits individually had no effects on S2 cell growth, as the number of viable cells on days 1 to 4 was comparable to that of the mock-treated S2 cells (Fig. 3). The only B subunit with potential redundancy is dB56. Notably, when both dB56-1 and dB56-2 were removed by co-RNAi, the increase in cell numbers over time was retarded, similar to the result seen in cells following RNAi of the A or C subunit (Fig. 3). Since RNAi of B56-1 and B56-2 individually had no effect, B56-2 is likely to be a bona fide PP2A subunit at least partially redundant with B56-1. Since RNAi of dB56-1 and of dB56-2 together had no effect on the protein levels of A and C subunits (data not shown), the failure of cell proliferation caused by loss of B56-1 + -2 is likely due to the down-regulation of specific B56-containing heterotrimers.

One potential cause of the failure of cell proliferation after RNAi of the B56 subunits could be a compensatory increase in other PP2A heterotrimers, i.e., increased PR55:PP2A and/or PR72:PP2A. Two results suggest that this is not the cause of growth failure and apoptosis (see below). First, the same phenotypes are caused by loss of A and C subunits, indicating that they are due to loss of phosphatase activity. Secondly, immunoblots examining PR55 protein abundance show no change following RNAi of B56-1 + -2 (data not shown).

RNAi of B56:PP2A induces apoptosis in S2 cells. Previous studies have implicated PP2A in the control of DNA replication (29, 44, 49). However, fluorescence-activated cell sorter analysis of dsRNA-treated S2 cells did not demonstrate any evidence of cell cycle arrest following RNAi of any of the PP2A subunits (data not shown). We next addressed whether the decreased accumulation was due to increased cell death. S2 cell morphology was examined after RNAi. As early as day 1 following the RNAi of dB56-1 + -2 A or C subunit, some cells began to exhibit membrane blebbing. By day 2, cells subjected to RNAi of the B56 subunits showed extensive membrane blebbing and apoptotic body formation. Nuclear condensation and DNA fragmentation commonly seen in apoptosis were also apparent following RNAi of the dB56-1 + -2 A or C subunit (Fig. 4D to F). These changes were absent in cells following mock treatment (Fig. 4A). Similar apoptotic changes were seen in S2 cells treated with the RNA synthesis inhibitor actinomycin D (Fig. 4C). RNAi of dPR55 (Fig. 4B), dPR72, or dB56-1 or dB56-2 subunits alone had no effect on cell morphology (data not shown). Therefore, RNAi of dB56-containing PP2A led to cell death in S2 cells with the characteristic morphological features of apoptosis.

Apoptosis induced by loss of B56:PP2A is mediated through the activation of DEVD-specific caspases and is blocked by RNAi of *Drosophila* caspases Dredd, Dronc, or Drice. Apoptosis in *Drosophila* requires activation of a series of initiator and effector caspases. To investigate whether loss of B56-regulated PP2A results in caspase activation, dsRNA-treated S2 cells were tested for caspase activity using a colorimetric caspase substrate. RNAi of the dB56-1 + -2 A or C subunit induced at least a fivefold increase in DEVD-specific caspase activity (Fig. 5A), while no significant increase in caspase activity was seen in cells treated with dsRNA for either dB56-1 or dB56-2 alone or for dPR72 combined with dPR55. As a positive control, RNAi-mediated loss of dIAP-1, a *Drosophila* inhibitor of apoptosis (17), induced the same level of caspase activation (Fig. 5A). Hence, the cell death caused by loss of B56-regulated PP2A is accompanied by caspase activation.

Prior studies have demonstrated that B56-containing PP2A heterotrimers are negative regulators of Wnt signaling in vertebrates (28, 37, 47) and, thus, that loss of B56-directed dPP2A heterotrimers could lead to accumulation of Armadillo (Arm), the *Drosophila* β-catenin homologue. Conceivably, excess Arm could contribute to apoptosis. To determine whether the loss of B56:PP2A-induced apoptosis is mediated through up-regulated Arm, we applied dsRNA of dAxin, a negative regulator of the Wnt signaling pathway, to S2 cells. As expected, RNAi of dAxin significantly up-regulated Arm protein levels (as did RNAi of dPP2A; data not shown). RNAi of dAxin had no effect on cell morphology or DEVD-specific caspase activity (Fig. 5A). RNAi of *arm* similarly did not stimulate apoptosis (data not shown). Dysregulated Wnt signaling is therefore unlikely to play a role in B56:PP2A-regulated apoptosis in cultured S2 cells.

Having found that caspase activation followed down-regulation of B56-containing dPP2A heterotrimers, we next asked if caspase activation was required for ensuing cell death. There are multiple caspases characterized in *Drosophila* (25). Among the *Drosophila* caspases, Dredd contains a death effector domain and Dronc contains a caspase recruitment domain, sug-

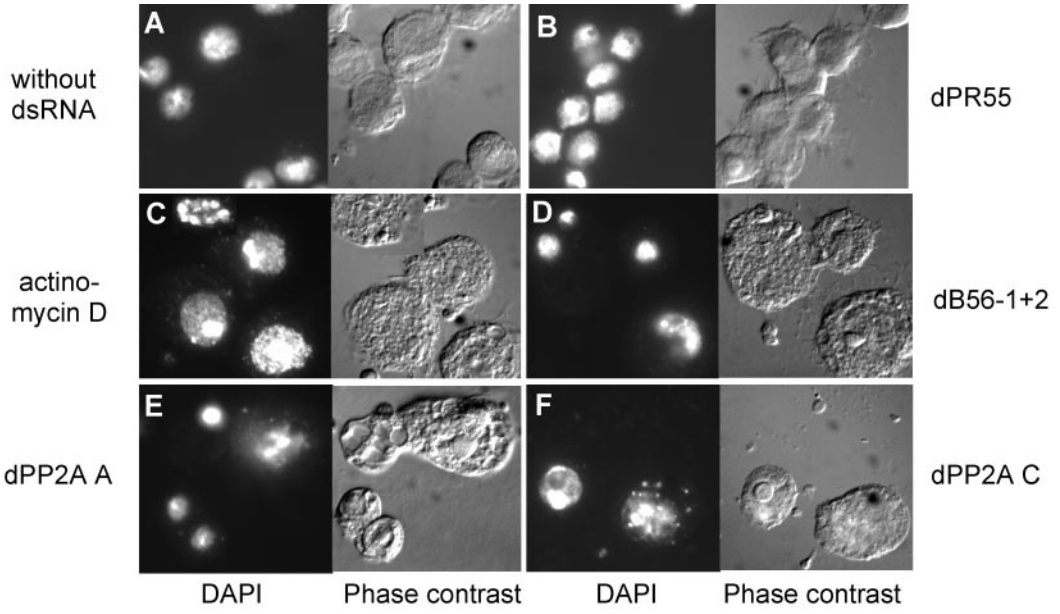

FIG. 4. Loss of B56:PP2A induces apoptotic morphology in S2 cells. S2 cells were fixed and stained with DAPI 4 days after treatment. Nuclear morphology was examined after DAPI staining, and cell morphology was examined by phase-contrast microscopy. Cells were either mock treated (A) or treated with dsRNA from dPR55 (B), 40 μM actinomycin D for only 15 h (C), dsRNA from both B56-1 and B56-2 (dB56-1+2) (D), dPP2A A subunit (E), or dPP2A C subunit (F). PP2A A, C, and combined B56 subunits produced morphological changes similar to those induced by actinomycin D. No morphological changes were seen following RNAi of individual B56 subunits or of PR55 or PR72 either alone or combined (data not shown).

gesting that they function as upstream initiator caspases (8, 35). The remaining caspases may act as effector caspases. One of these effector caspases, Drice, has previously been shown to be required for apoptotic activity in S2 cell extracts (15). RNAi of individual caspases was performed in combination with RNAi of PP2A subunits (Fig. 5B to F). Four days after the addition of dsRNA, the extent of cell death was judged by both cell morphology and cell viability. RNAi of either dB56-1 + -2 or dPP2A C led to a significant decrease in the number of viable cells (Fig. 5B) and to characteristic changes in cell morphology (membrane blebbing and apoptotic body formation) similar to those seen when dIAP-1 was knocked down (Fig. 5C and E and data not shown). RNAi of Drice, Dredd, or Dronc combined with RNAi of B56-1 + -2 or with C subunit rescued cell proliferation (Fig. 5B) and prevented apoptotic changes in cell morphology (Fig. 5D). This effect was highly specific, since co-RNAi of eight unrelated genes did not block the apoptosis induced by RNAi of B56-1 + -2 (data not shown). These data indicate that the apoptosis induced by loss of B56-targeted PP2A in S2 cells requires the activation of caspases and that Dredd, Dronc, and Drice lie downstream of B56 and dPP2A.

Knockdown of B56:PP2A induces extensive apoptosis in *Drosophila* embryogenesis. To extend these findings to whole animals, we assessed the effect of disrupting PP2A function in vivo. *Drosophila* embryos were injected with dsRNA samples prior to cellularization, and their developmental progression was monitored (Fig. 6A). Sixty-three percent of embryos injected with a buffer control ($n = 84$) developed normally and completed germ band extension within 6 to 8 h after injection. In contrast, injection of embryos with dsRNA corresponding to *dIAP-1* led to widespread induction of apoptosis and 95% of embryos aborted development prior to the extended-germband stage of embryogenesis ($n = 40$). Cuticles derived from injected embryos were devoid of all pattern elements (data not shown). dsRNA-mediated inhibition of dB56-1 + -2 functions ($n = 124$) or of PP2A A subunit function ($n = 46$) similarly disrupted embryonic development in a large proportion of injected embryos. In both cases ~70% of injected embryos aborted development prior to the extended-germ-band-stage. In fact, these embryos displayed none of the morphological markers normally associated with a wild-type developmental program. We next determined whether the developmental block induced by *dB56-1* and *-2* dsRNAs was due to induction of ectopic apoptosis. Acridine orange was used to visualize the nuclei of dying cells in injected embryos. Whereas buffer-injected control embryos displayed characteristic patterns of cell death (2), ectopic cell death was clearly evident in embryos injected with dsRNAs corresponding to *dB56-1* and *-2* (Fig. 6B and C). This effect was highly specific, since RNAi of four unrelated transcripts had no effect on cell death or early embryonic development (data not shown).

Since RNAi of the B56 subunits led to both cell death and aberrant development, the B56 subunits could be performing two distinct functions in early embryogenesis. To determine if excess apoptosis was the primary defect, embryonic development was assessed in embryos coinjected with *dB56-1* and *-2* and *Drice* dsRNAs ($n = 104$). Loss of the caspase Drice led to a near-total rescue of embryonic development to the extended-germ-band stage as well as to a loss of apoptosis in injected embryos (Fig. 6D). Taken together, these data are consistent with our S2 cell culture studies and strongly suggest that the

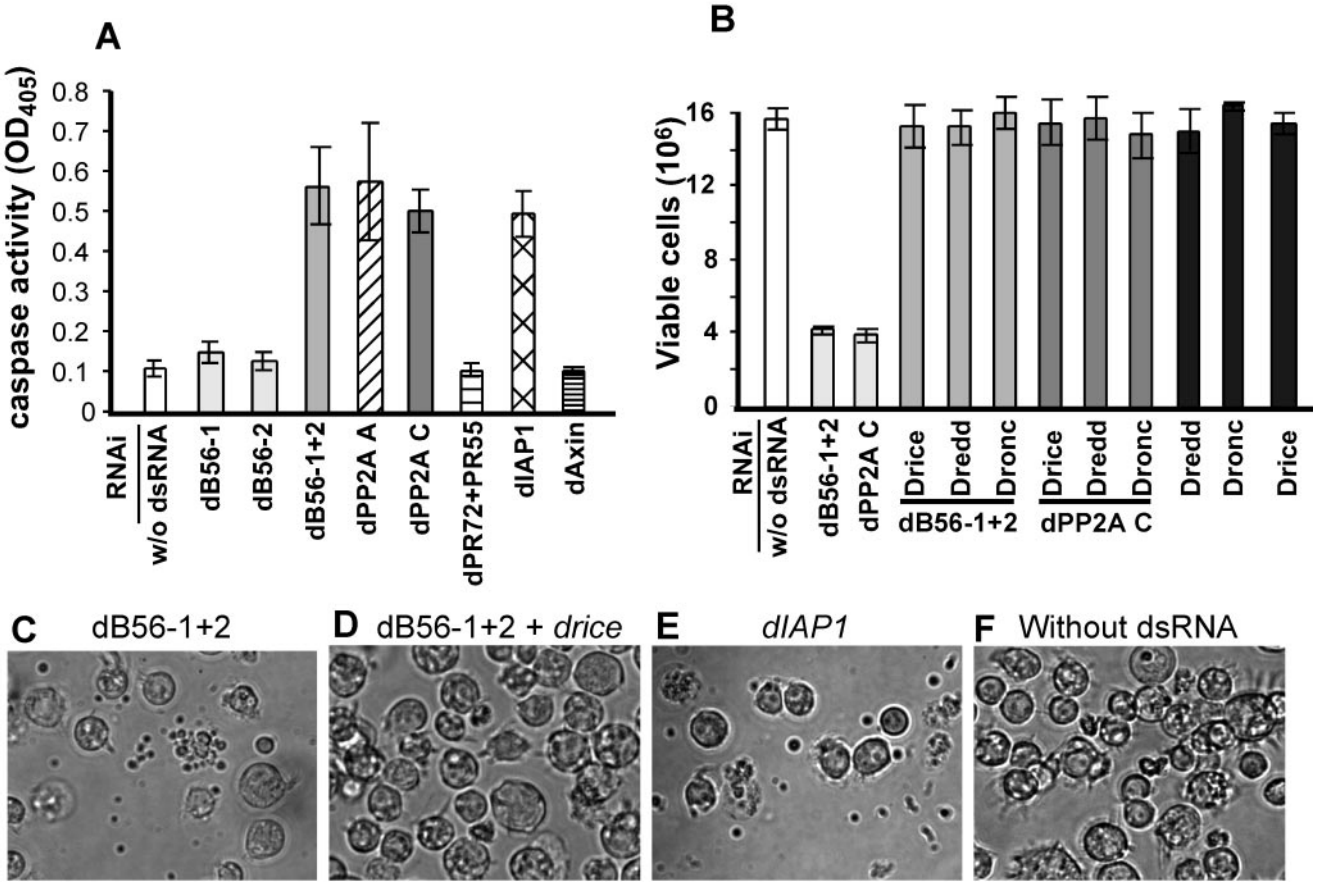

FIG. 5. Apoptosis induced by loss of B56-regulated PP2A is accompanied by activation of DEVD-specific caspases and inhibited by RNAi of caspases Dredd, Dronc, and Drice. (A) Caspase activation following RNAi of B56-regulated PP2A. DEVD-specific caspase activity in the cell extracts was assayed 4 days after RNAi of the indicated genes. OD_{405}, optical density at 405 nm. (B) Caspases Dredd, Dronc, and Drice are required for the cell death that follows RNAi of B56-targeted PP2A. Viable S2 cells were counted 4 days following RNAi of the indicated genes. (C to F) RNAi of dB56-1 and -2 (C) induces apoptotic morphology in S2 cells 4 days after application of the dsRNA, an effect blocked (D) by addition of dsRNA-targeting *drice*. Similar apoptotic changes were seen with RNAi of *dIAP-1* (E). Control cell morphology is shown in panel F.

developmental failure following RNAi of *B56-1 + -2* in early *Drosophila* embryos is due to the effects of B56 on apoptosis.

B56:PP2A acts upstream of Dark, Reaper, Hid, and dp53. The above analysis suggests that dPP2A normally acts upstream of caspase activation and that one of its normal functions is to prevent apoptosis, since loss of B56-targeted PP2A led to apoptosis and a failure of normal embryogenesis. To epistatically position B56-regulated PP2A in the apoptotic pathway, upstream regulators of cell death were knocked down in combination with PP2A RNAi and the effect on caspase activation and cell morphology was determined. The *Drosophila* homologue of *apaf1*, *dark*, is postulated to bind to cytochrome *c* released from mitochondria and to activate initiator caspases. RNAi of *dApaf1* blocked both caspase activation (Fig. 7A) and the morphological changes (Fig. 7B and C) induced both by RNAi of B56-1 + -2 and of the C subunit. Thus, B56-containing PP2A is upstream of *dApaf1*.

Apoptosis in *Drosophila* is controlled by three genes, *reaper*, *hid*, and *grim*. Because the expression of these genes may be low until apoptotic stimuli, we first assessed the effect of RNAi on their mRNA levels. Levels of *dp53*, *rpr*, *hid*, and *grim* mRNA were detectable in log-phase S2 cells and were significantly though not completely reduced by RNAi (Fig. 7D). RNAi of PP2A C alone or of B56-1 + -2 combined led to caspase activation, as expected (Fig. 7E). Co-RNAi of *rpr*, *hid*, and *dp53* substantially inhibited that caspase activation (Fig. 7E). RNAi of *grim*, while effective in decreasing *grim* mRNA abundance, had no effect on caspase activation induced by RNAi of PP2A subunits (Fig. 7E). The data suggest that B56-containing PP2A is upstream both of dp53 and its downstream targets *rpr* and *hid*.

DISCUSSION

This study identifies PP2A and specifically a PP2A heterotrimer containing a B56-targeting subunit as a critical element in prevention of apoptosis in cultured cells and embryos in *Drosophila*. Knockdown of PP2A A or C or both B56-targeting subunits led to development of the morphological and biochemical changes characteristic of programmed cell death, including membrane blebbing, nuclear condensation, apoptotic body formation, and caspase activation. Loss of B56-regulated PP2A activated an apoptotic program in *Drosophila* embryos as well, as evidenced by RNAi-induced acridine orange stain-

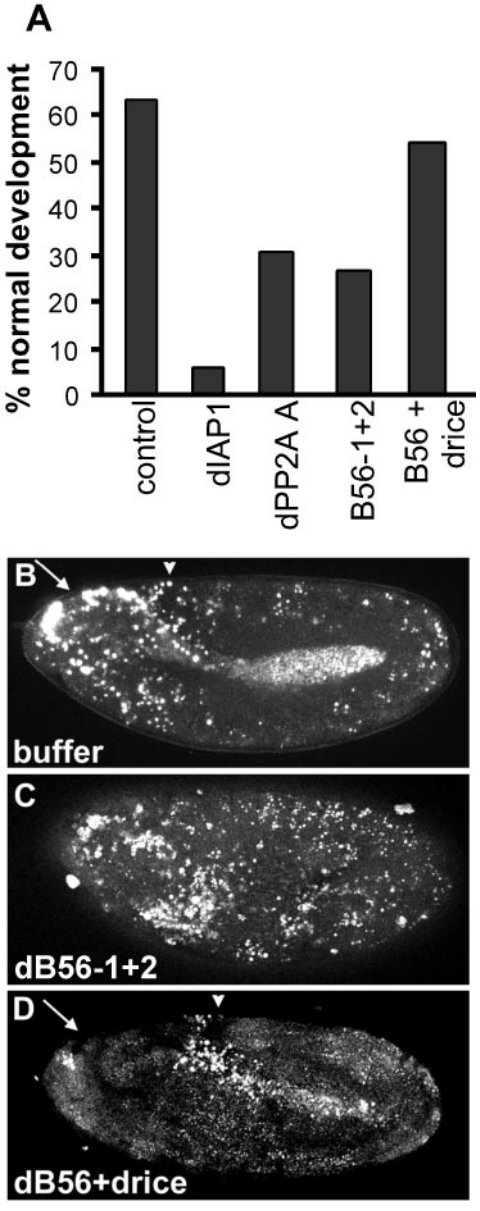

FIG. 6. Disruption of dB56-1and dB56-2 function leads to ectopic cell death and developmental arrest. (A) Graphic representation of embryonic viability following RNAi. The decrease in viability of *dB56-1* and *-2* dsRNA -injected embryos is statistically different from that of buffer-injected control embryos using *chi*-square analysis ($P = 1.58 \times 10^{-7}$). Viability of *dB56-1* and *-2 Drice* dsRNA-injected embryos is not significantly different from that of buffer-injected controls but is significantly different from that of *dB56-1+ -2*-coinjected embryos ($P = 2.69 \times 10^{-5}$). (B) Buffer-injected control embryos at stage 10 or 11 displayed wild-type patterns of development. Notice wild-type head (arrow) and extended-germ-band (arrowhead) structures as well as wild-type patterns of cell death. Cell death was visualized after acridine orange staining; in this and subsequent images, dying cells appear white. (C) Injection of 2.5 μM *dB56-1* and 2.5 μM *dB56-2* dsRNAs led to aborted embryonic development and ectopic cell death. (D) Coinjection of 2.5 μM *dB56-1* and *-2* dsRNAs with 2.5 μM *Drice* dsRNA rescues the ectopic cell death phenotype and restores wild-type morphology to injected embryos. Identical results were seen in embryos injected with a 1.25 μM concentration of each dsRNA.

ing, failure of germ band extension, and rescue of both phenotypes by RNAi of a downstream caspase. Epistasis analysis placed B56-regulated PP2A upstream of *dp53*, *rpr*, *hid*, and *dark*, suggesting that loss of the specific phosphatase activates cell death pathways dependent on the damage-sensing function of dp53.

The ability of PP2A to promote cell survival appears to require the association of at least one of the two B56-targeting subunits in the PP2A heterotrimer. Knockdown of a single B56 gene had no effect on cell survival. Supporting this, Berry and Gehring have reported recently that knockdown of B56-1/B' resulted in embryos without salivary glands (4). Two other targeting subunits exist in *Drosophila*, B/PR55 and PR72, and loss of one or both of these had no effect on S2 cell proliferation or indicators of apoptosis. While these subunits have been implicated in chromosome segregation and cell cycle progression, we did not detect any alteration in these processes (30, 49). It is possible that our assays were not sufficiently sensitive to detect more subtle changes in mitotic segregation and growth rates. Alternatively, homology searches may have failed to identify additional B subunits redundant in these functions, or the protein half-lives may have been sufficiently long to allow a small amount of protein to persist and carry out essential functions. However, the substantial decrease in A and C proteins when the four identified B subunits were knocked down strongly suggests that the major B subunit genes have been identified.

The simplest model to explain these results is that the B56 subunits of PP2A target the heterotrimer to dephosphorylate an antiapoptotic substrate or substrates in S2 cells and early embryos (Fig. 8). Loss of both B56 subunits or loss of the A or C subunits leads to hyperphosphorylation of this substrate. Accumulation of the hyperphosphorylated substrate (or loss of the unphosphorylated substrate) may directly trigger p53-dependent apoptosis or may disrupt signal transduction upstream of dp53 sufficiently to trigger apoptosis. It is notable that loss of B56 regulatory subunits triggers apoptosis, while loss of other B subunits does not. Hence, it is likely that there are only a limited number of PP2A substrates whose altered phosphorylation leads to programmed cell death. This is consistent with the hypothesis that apoptosis in this system is not a nonspecific result of increased phosphorylation of multiple proteins but rather a consequence of hyperphosphorylation of a key substrate(s).

Epistasis analysis places B56:PP2A upstream of *dark*, *reaper*, *hid*, and *dp53*. The role of Dark in the activation of caspases is well conserved in *Drosophila.* Like Apaf1 in mammalians, in response to proapoptotic signaling, Dark interacts with cytochrome *c*, which is released from mitochondria during the apoptotic response (21, 35, 36). Cytochrome *c* release in mammals is mediated by proapoptotic Bcl-2/BAX-related proteins. However, RNAi of the only Bcl-2/BAX-related gene identified to date in *Drosophila*, a proapoptotic factor variously named dBOK, dBorg, or Debcl, did not block B56-knockdown-induced apoptosis (data not shown). Activated Dark may be a required cofactor for the activation of Dredd and Dronc but may not be the direct initiator of cell death. RNAi of *rpr* and *hid* but not of *grim* substantially inhibited apoptosis induced by loss of B56:PP2A. Reaper, Hid, and Grim proteins induce apoptosis at least in part by binding to and inactivating the

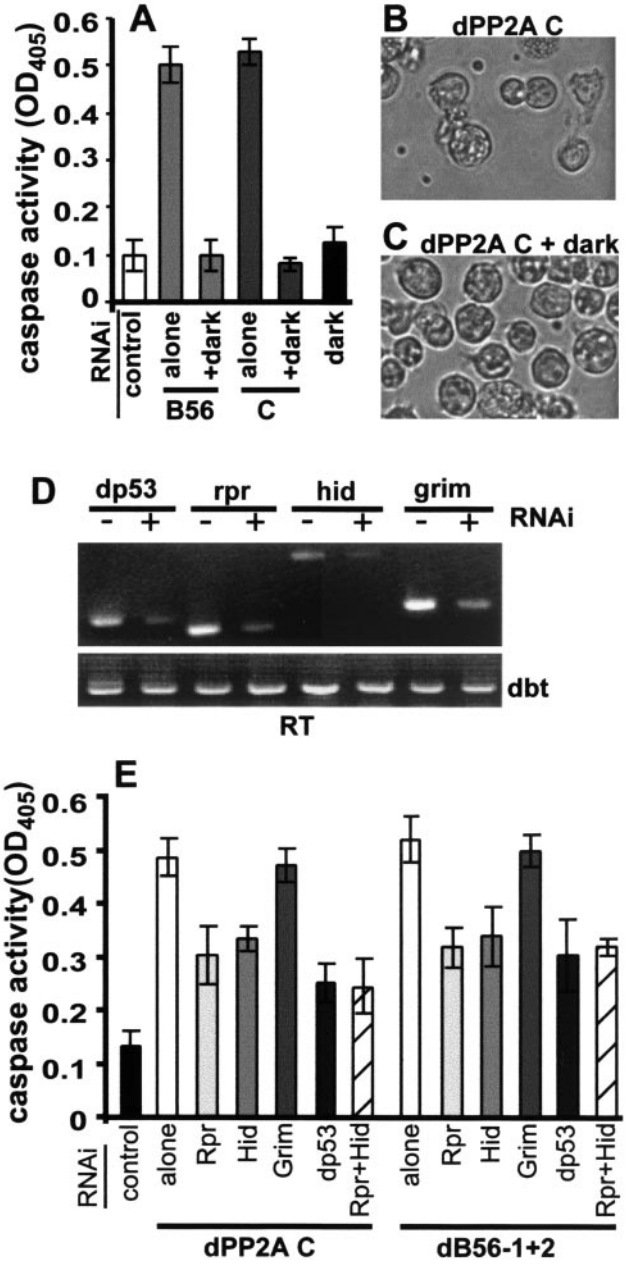

FIG. 7. B56-targeted PP2A acts functionally upstream of Dark/dApaf1, Reaper, and Hid, and dp53. (A) Loss of *dark* abolishes B56:PP2A-regulated activation of DEVD-dependent caspases. Caspase activity was determined 4 days following RNAi of the indicated genes. (B) RNAi of dPP2A C induces morphological changes characteristic of apoptosis. S2 cells were examined by phase-contrast microscopy 4 days following RNAi of dPP2A C. (C) Addition of dsRNA for *dark* blocks the morphology changes. Similar results were seen with dB56 subunits (data not shown). (D) Partial knockdown of upstream activators of apoptosis by RNAi in S2 cells. dsRNA derived from *reaper*, *hid*, *grim*, and *dp53* was added to S2 cells as described earlier, and mRNA levels were assessed by RT-PCR 4 days later. RT-PCR (RT) against *dbt* was performed on each sample as a control for RNA recovery. (E) RNAi of *rpr*, *hid*, or *dp53* but not of *grim* partially suppresses B56:PP2A-regulated activation of DEVD-specific caspases. OD_{405}, optical density at 405 nm.

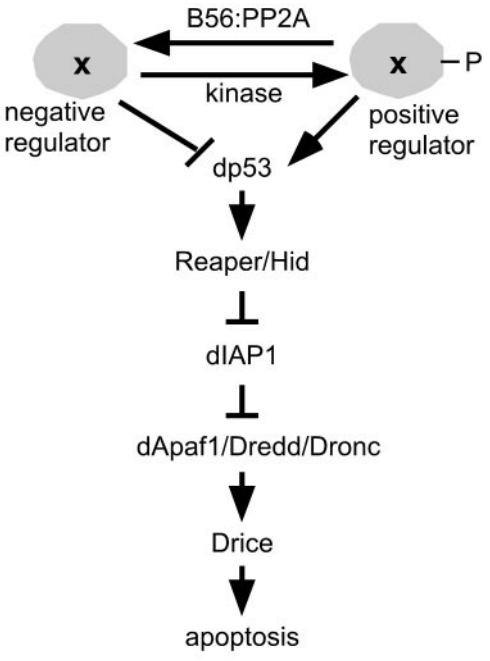

FIG. 8. A model for B56:PP2A-regulated apoptosis in *Drosophila*. B56-regulated PP2A normally maintains an upstream factor (X) in the dephosphorylated state. In the absence of specific PP2A activity, X becomes hyperphosphorylated, leading to p53-dependent induction of apoptosis.

antiapoptotic *Drosophila* homologues of the IAPs. The requirement for both *hid* and *rpr* for apoptosis is consistent with the findings in some *Drosophila* tissues that coordinated expression of both genes is required for programmed cell death (46, 50). While up-regulation of *rpr* and *hid* expression is seen in some tissues preceding apoptosis, we found that these genes were already expressed in S2 cells and that RNAi-induced apoptosis did not further increase their mRNA abundance (data not shown). Thus, loss of specific B56-targeted PP2A activity may up-regulate the activity of these proteins without induction of gene expression. The effect of RNAi on *dp53*, *hid*, *grim* and *rpr* mRNA levels was only partial. This incomplete inhibition of caspase activation after knockdown of B56-targeted PP2A could be due either to incomplete loss of *dp53*, *hid*, and *rpr* function, or alternatively, other proapoptotic pathways may also be involved.

Phosphorylation of Hid by MAP kinase has been reported to inhibit apoptosis, while PP2A knockdown activates apoptosis (3). Hence, B56-regulated PP2A is unlikely to be the Hid phosphatase. Loss of *dp53* inhibits apoptosis induced by loss of B56:PP2A, suggesting that B56:PP2A acts upstream of *dp53*. *dp53* is required for radiation-mediated apoptosis, and one of the transcriptional targets of *dp53* is *rpr*. Phosphorylation of mammalian p53 is a common event in the p53-mediated response to DNA damage, and loss of a p53 phosphatase might be expected to mimic the activation of a p53 kinase. In fact, p53 becomes hyperphosphorylated in mouse 3T3 cells treated with okadaic acid (48). However, dp53 does not retain many of the phosphorylation sites identified in mammalian p53. Furthermore, in preliminary studies, RNAi of *Drosophila* homologues

of p53 kinases such as ATM and JNK did not block the B56-knockdown induced apoptosis (data not shown). Further study is needed to determine if p53 is a direct target of B56-directed PP2A and if PP2A knockdown alters p53 protein abundance and its phosphorylation state.

Finally, we note that this study provides in vivo evidence that *Drosophila* PP2A is primarily a heterotrimer and that free A:C heterodimers are unstable or insoluble in the absence of a targeting subunit. RNAi of the four identified B subunits did not completely remove all A and C subunit protein, which could be due to the persistence of A:C dimers, failure to eliminate all B subunit protein, or the presence of additional lower-abundance PP2A binding proteins able to stabilize the A:C dimer. However, the simplest explanation of the data is that the knockdown of B subunits leads to instability of A:C dimers and that the identified B subunits make up the large majority of PP2A binding proteins in S2 cells. This result is in contrast to findings of AC dimers in mammalian cells (24). It may be that the stability of *Drosophila* PP2A differs significantly from that of mammalian PP2A dimers.

In summary, the present study demonstrates that B56-associated PP2A is required for S2 cell survival, as its absence leads to dp53-dependent apoptosis. Further study is required to identify the B56:PP2A substrates, whose altered phosphorylation triggers apoptosis.

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