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A genome-wide RNAi screen reveals multiple regulators of caspase activation

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Apoptosis is an evolutionally conserved cellular suicide mechanism that can be activated in response to a variety of stressful stimuli. Increasing evidence suggests that apoptotic regulation relies on specialized cell death signaling pathways and also integrates diverse signals from additional regulatory circuits, including those of cellular homeostasis. We present a genome-wide RNA interference screen to systematically identify regulators of apoptosis induced by DNA damage in Drosophila melanogaster cells. We identify 47 double-stranded RNA that target a functionally diverse set of genes, including several with a known function in promoting cell death. Further characterization uncovers 10 genes that influence caspase activation upon the removal of Drosophila inhibitor of apoptosis 1. This set includes the Drosophila initiator caspase Dronc and, surprisingly, several metabolic regulators, a candidate tumor suppressor, Charlatan, and an N-acetyltransferase, ARD1. Importantly, several of these genes show functional conservation in regulating apoptosis in mammalian cells. Our data suggest a previously unappreciated fundamental connection between various cellular processes and caspase-dependent cell death.

Introduction

Apoptosis is an evolutionally conserved cell death pathway that regulates development and tissue homeostasis. Caspases, a family of cysteine proteases, play a critical role in mediating the execution of apoptosis. Although CED-3 is the sole caspase required for programmed cell death in Caenorhabditis elegans, multiple caspases mediate apoptotic cell death in flies and mammals. In these systems, the activation of upstream initiator caspases in response to proapoptotic signals leads to activation of the downstream executioner caspases. Although the core apoptotic pathway has been studied extensively, many aspects of the signaling networks that control the cellular decision to undergo apoptosis remain unknown. Complex biological processes have been dissected successfully using genome-wide RNAi screens in Drosophila melanogaster cells. In this study, we describe the isolation of 10 genes, including the apical caspase Dronc, that are required for full caspase activation in response to DNA damage. Surprisingly, we discovered that Charlatan (Chn), a regulator of neuronal cell differentiation (Tsuda et al., 2006), and ARD1, an N-acetyltransferase involved in cell fate specification (Whiteway and Szostak, 1985), regulate caspase activation. Importantly, we show that certain fly genes are functionally conserved as modifiers of caspase activation in the mammalian system. Our screen implicates Chn and ARD1 as a molecular link between cellular differentiation and apoptosis.

Results and discussion

To determine the feasibility of an RNAi approach in identifying apoptotic regulators, we tested whether the knockdown of Dcp-1, a downstream effector caspase functionally similar to mammalian caspase-3, protects against DNA damage–induced apoptosis in Drosophila embryonic hemocyte Kc cells (Song et al., 2000). We used a topoisomerase II inhibitor, doxorubicin (dox), to induce dose-dependent cell death (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200708090/DC1) that can be suppressed by z-VAD.fmk treatment (Fig. S1 B). As expected, dcp-1 RNAi partially protected cells from apoptosis induced by dox (Fig. S1 C), which is consistent with previous observations (Xu et al., 2006). We conclude that dox induces caspase-dependent cell death in Kc cells that can be suppressed by a specific double-stranded RNA (dsRNA) and, therefore, represents a suitable system for identifying modulators of apoptosis.
Figure 1. **Identification and confirmation of dsRNAs that provide protection against DNA damage–induced apoptosis.** (A) dsRNAs were screened in duplicate plates for protection against dox treatment. The threshold for a hit was set at a z score of at least 2 for both plates (above blue dashed line). The plot represents the z score analysis of several plates that were enriched for the genes shown in Table I (red diamonds). (B) dsRNAs identified in the primary screen were individually tested, and those that provided statistically significant protection against dox in at least two independent experiments are shown (P < 0.05; t test). The graph represents a pool of 10 sample plates, and the error bars represent the SD of dsRNAs tested in triplicate. (C) This graph depicts the functional distribution of genes shown in B according to information provided by FlyBase (version FB2006_01).
A diverse set of dsRNAs protects fly cells from dox-induced apoptosis

To identify dsRNAs that inhibit DNA damage–induced apoptosis in Kc cells, we performed a high-throughput screen using an established genome-wide Drosophila RNAi library that targets 19,470 genes (Boutros et al., 2004). 81 dsRNAs resulted in a z score >2, which was the threshold for defining a hit in our primary screen (Fig. 1 A). To eliminate dsRNAs that directly enhanced cellular ATP levels, the effect of dsRNAs on ATP levels was measured in the rescreen. We verified that 62 dsRNAs specifically protected cells against dox-induced apoptosis (Fig. 1 B). To minimize off-target effects, we further examined any dsRNA with at least 19-nucleotide sequence identity with an off-target gene (Echeverri et al., 2006; Ma et al., 2006) by testing alternative dsRNAs distinct from the original targeting sequence for protection against cell death induced by dox treatment (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200708090/DC1) and for caspase suppression induced by Drosophila inhibitor of apoptosis 1 (diap1) RNAi treatment as described in Fig. 3 (Fig. S2 B). Any dsRNA for a given gene failing to provide significant protection (P < 0.05) in either of these assays was eliminated, resulting in a final set of 47 genes (Fig. 1 B and Table S1).

The identification of three known regulators of cell death validates the ability of our screen to uncover genes required for promoting apoptosis. Silencing of Dronc provided maximal protection against dox treatment (Fig. 1 B), which is consistent with its role as the main checkpoint for apoptosis in the fly (Chew et al., 2004; Daish et al., 2004). In addition, knockdown of the edcsyne-induced protein Eip63F-1 provided the fourth strongest protection against DNA damage. The increased expression of Eip63F is detected in the premetamorphic salivary gland of Drosophila larvae, immediately before the edcsyne-mediated induction of massive autophagic cell death (Gorski et al., 2003; Rusten et al., 2004). Lastly, our screen isolated Jra, the Drosophila orthologue of a known proapoptotic mammalian transcriptional factor, c-Jun, as a mediator of DNA damage–induced apoptosis (Verheij et al., 1996).

Approximately 85% of the genes identified in the RNAi screen are characterized genes of known function or contain well-conserved functional domains, which regulate a wide range of cellular processes, including signaling, metabolism, and transcription, whereas the remaining 15% of the genes have no known functional domains (Fig. 1 C). Altogether, our RNAi screen implicates cell death genes (three), signaling molecules (13), metabolic regulators (10), metabolite transport factors (four), genes involved in ER/Golgi trafficking (four), chromatin/transcription regulators (two), RNA-processing factors (two), structural and cytoskeletal proteins (two), and genes of unknown function (seven) in mediating DNA damage–induced apoptosis (Table S1). Strikingly, 20% of the genes are directly involved in cellular metabolic processes (Fig. 1 C), supporting an earlier proposal that the cellular metabolic state critically influences the threshold for induction of apoptosis (Hammerman et al., 2004). To investigate where these genes operate in the apoptotic pathway, we conducted specific enzymatic and epistatic assays in fly and mammalian cells.

Identification of genes involved in caspase-dependent cell death

Next, we classified the genes that are specifically involved in caspase-dependent cell death. We observed the substantial induction of caspase activity 8 h after dox treatment, preceding detectable cell death (Fig. S1, A and D). Any RNAi suppressing this activity implicates the target gene in early regulation of caspase activation. In addition to dcp-1 RNAi, knockdown of dronc and jra significantly (P < 0.05) suppressed caspase-3/7–like activity in the presence of dox, whereas the negative control, RNAi against calpain A, a calcium-dependent cysteine protease, did not affect this pathway (Fig. 2 A). We expanded this analysis to all of the genes identified in the initial RNAi screen and discovered 20 dsRNAs that suppressed caspase activation induced by DNA damage (Table S1). Interestingly, as shown in Fig. 2 B, 12 of these genes were found to be epistatic to diap1, as discussed in the next section.
Identification of genes epistatic to DIAP1

Next, we performed \textit{diap1} epistatic analysis to further categorize the genes. DIAP1, the fly orthologue of the mammalian inhibitors of apoptosis proteins, is a direct inhibitor of caspases, and deficiency in DIAP1 leads to rapid caspase activation and apoptosis in vivo (Wang et al., 1999). Thus, apoptosis induced by the loss of DIAP1 presents an alternative apoptotic assay independent of DNA damage. Silencing of genes that regulate activation of the core apoptotic machinery may provide protection against apoptosis induced by both DNA damage and the loss of DIAP1. RNAi against \textit{dcp-1} partially suppressed cell death induced by the depletion of DIAP1 in \textit{Kc} cells (Fig. 3 A). Also, \textit{dronc} RNAi potently protected cells against apoptosis induced by deficiency in DIAP1 as reported previously (Meier et al., 2000; Chai et al., 2003). Altogether, 32 of the genes confirmed from our primary screen provided significant (P < 0.05) protection against cell death induced by the silencing of DIAP1 (Table S1 and Fig. 3 B).

Interestingly, 12 dsRNAs suppressed caspase-3/7–like activity after dox treatment and protected against cell death induced by \textit{diap1} RNAi, suggesting that these genes are required for apoptosis induced by multiple stimuli (Table I). To confirm that these genes are necessary for the full activation of caspases, we determined whether these dsRNAs could suppress spontaneous caspase activity induced by \textit{diap1} RNAi. We observed maximal induction of caspase activity by \textit{diap1} RNAi after 24 h, and this effect was completely suppressed by dsRNA against \textit{dcp-1} (Fig. S1 E). Importantly, ablating 10/12 dsRNAs resulted in the significant (P < 0.05) suppression of caspase activity compared with \textit{diap1} RNAi only (Fig. 3 C).

In addition to \textit{dronc} RNAi, dsRNAs targeting \textit{chn} and \textit{dARD1} provided the strongest suppression of spontaneous caspase activity. Consistent with our observation that RNAi against \textit{chn} protects against DNA damage–induced cell death, the mammalian orthologue neuron-restrictive silencer factor (NRSF)/RE1-silencing transcription factor (REST) was recently identified as a candidate tumor suppressor in epithelial cells (Westbrook et al., 2005). Previous work indicates that Chn and NRSF/REST function as a transcriptional repressor of neuronal-specific genes.
We assessed the ability of siRNAs targeting a gene of interest to protect against DNA damage in HeLa cells. As a positive control, cells were transfected with siRNAs targeting Bax or Bak, two central regulators of mammalian cell death (Wei et al., 2001; Zong et al., 2001). Indeed, silencing of Bax or Bak resulted in significant protection (P < 0.05) against dox-induced cell death (Fig. 4 A). We observed that plk3 RNAi provided partial protection against dox treatment, which is consistent with previous studies implicating Plk3 in stress-induced apoptosis (Xie et al., 2001a,b; Bahassi et al., 2002). Interestingly, the knockdown of hARD1 dramatically enhanced cell survival in the presence of dox to levels similar to that of Bak. This protective effect was also evident at the morphological level. In cells transfected with a nontargeting control siRNA, dox treatment resulted in typical apoptotic morphology, including cell rounding and membrane blebbing (Fig. 4 B). In direct contrast, cells transfected with siRNAs against hARD1 maintained a normal and healthy morphology and continued to proliferate in the presence of dox.

To further explore the significance of our findings, we examined whether silencing the mammalian orthologues of the fly genes identified from the RNAi screen confers protection against dox-induced cell death in mammalian cells. We selected a set of mammalian orthologues that are believed to be nonredundant. The list includes the orthologues of dMiro, which functions as a Rho-like GTPase; dARD1, which functions as an N-acetyltransferase; CG12170, which functions as a fatty acid synthase; and Chn, which functions as a transcriptional repressor (RHO1, hARD1, OXSM, and REST, respectively; FlyBase). In addition, we tested Plk3, a mammalian orthologue of Polo, as dsRNA targeting polo potently protected against dox treatment (Fig. 1).
Figure 4. **hARD1 is required for caspase activation in response to DNA damage in mammalian cells.** (A) HeLa cells were transfected with a pool of four siRNAs as indicated followed by treatment with 1.25 μg/ml dox for 20 h. This graph is representative of three independent experiments. (B) HeLa cells were transfected with siRNAs as indicated and treated with 1.25 μg/ml dox followed by a 12-h incubation. Cells were visualized by bright-field microscopy, and representative images of samples tested in triplicate are shown. (C) HeLa cells were transfected with siRNAs as indicated and were treated with 5 μg/ml dox for 8 h. This graph is representative of three independent experiments. (D) HeLa cells were transfected with a pool of siRNAs or individual siRNAs targeting hARD1 as indicated and were treated with 5 μg/ml dox for 8 h. Error bars represent SD. (E) HeLa cells were transfected with siRNAs as indicated and treated with 5 μg/ml dox for 8 h. Note that RNAi against hARD1 completely inhibited the cleavage of caspase-2, caspase-9, and caspase-3 compared with nontargeting siRNA (arrows). This experiment is representative of two independent experiments. (F) HeLa cells were transfected with siRNAs as indicated and treated with 5 μg/ml dox for 8 h. Asterisks represent nonspecific bands recognized by the indicated antibody. This experiment is representative of two independent experiments. (A, C, and D) *, P < 0.05; t test.
cleavage in reconstituted hARD1 knockdown cells. Because six out of six siRNAs against hARD1 provided strong protection against DNA damage–induced apoptosis and complementation of hARD1-sensitized cells to caspase activation, we conclude that the functional role of ARD1 for dox-induced apoptosis is evolutionarily conserved from Drosophila to mammals.

In contrast to our results, Arnesen et al. (2006) reported that hARD1 is necessary to maintain cell survival. One possible explanation for this discrepancy can be attributed to the inherent differences between the siRNAs used in this study and that used by Arnesen et al. We observed that two out of two siRNAs used in the Arnesen et al. (2006) study resulted in a decrease in cell survival in the absence of stress signal, whereas none of the siRNAs tested as such had a negative effect on cell survival (Fig. S2 D).

In summary, we used an unbiased RNAi screening platform in Drosophila cells to identify genes involved in promoting DNA damage–induced apoptosis. We isolated 47 dsRNAs that suppress cell death induced by dox. These genes encode for known apoptotic regulators such as Drone, the Drosophila orthologue of the known proapoptotic transcriptional factor c-Jun, and an edcsone-regulated protein, Eip63F-1, thereby validating our primary screen. Furthermore, our study implicates a large class of metabolic genes that were previously not suspected to have a role in modulating caspase activation and apoptosis, such as genes involved in fatty acid biosynthesis (CG11798), amino acid/carbohydrate metabolism (CG31674), citrate metabolism (CG14740), complex carbohydrate metabolism (CG10725), and ribosome biosynthesis (CG6712). These results support an earlier proposal (Hammerman et al., 2004; Nutt et al., 2005) that the cellular metabolic status regulates the threshold for activation of apoptosis and thus plays a critical role in the decision of a cell to live or die.

Of particular interest is the identification of ARD1. We present evidence that RNAi against ARD1 provides protection against cell death and leads to the suppression of caspase activation induced by DNA damage in fly cells and HeLa cells. Furthermore, deficiency in dARD1 renders fly cells resistant to the spontaneous caspase activity and cell death associated with loss of Diap1. Importantly, we provide substantial evidence that hARD1 is required for caspase activation in the presence of DNA damage in mammalian cells. Cleavage of initiator and executioner caspases are suppressed in hARD1 RNAi cells treated with dox, suggesting that hARD1 functions further upstream of caspase activation, and the complementation of hARD1 knockdown cells restores caspase-3 cleavage. These data indicate that ARD1 is necessary for DNA damage–induced apoptosis in flies and mammals.

ARD1 functions in a complex with N-acetyltransferase to catalyze the acetylation of the N-terminal residue of newly synthesized polypeptides and has been implicated in the regulation of heterochromatin, DNA repair, and the maintenance of genomic stability in yeast (Mullen et al., 1989; Aparicio et al., 1991; Ouspenski et al., 1999; Wilson, 2002; Bilton et al., 2006). These studies suggest that ARD1 may be involved in regulating an early step in response to DNA damage. We anticipate that future studies will focus on determining whether ARD1 functions in similar processes in mammals. The diversity of genes identified in our screen illustrates the complex cellular integration of survival and death signals through multiple pathways.

Materials and methods

Tissue culture

Drosophila Kc167 cells were maintained in Schneider’s Drosophila medium with 10% FBS in the presence of penicillin and streptomycin (Invitrogen). HeLa cells were maintained in DME with 10% newborn calf serum in the presence of penicillin and streptomycin (Invitrogen) and were passaged for no more than 6 wk.

RNAi screening

Drosophila Kc cells (105/well in 384-well plates) were transfected with prealiquoted dsRNAs (0.5 μg/well) followed by a 48-h incubation to allow for protein turnover. Subsequently, cells were treated with 5 μg/ml dox (Sigma-Aldrich) for an additional 48-h incubation. Cell viability was determined by measuring cellular ATP levels (CellTiter-Glo luminescent cell viability assay; Promega). Positive hits for this screen were defined as dsRNAs that increased cellular ATP levels by at least two units of SD above the mean level of the plate, a z score of two, in duplicate.

Rescreen

Confirmation of these hits was performed using newly synthesized dsRNAs. cDNA was amplified from fly genomic DNA using primers optimally designed for RNAi (sequences provided by N. Ramadon, Drosophila RNAi Screening Center, Harvard Medical School, Boston, MA). dsRNA synthesis was conducted according to the manufacturer’s recommended protocol (MegaScript; Ambion). Kc cells (2 × 105/well in 96-well plates) were transfected with dsRNAs (1 μg/well) followed by treatment with dox. Each dsRNA was tested in triplicate and in at least two independent experiments. Cell viability was determined as a ratio between ATP levels of cells treated with a given dsRNA in the presence of dox and control cells treated with the dsRNA only. Statistically significant protection (P < 0.05) against dox-induced cell death was determined by comparing the cell viability of cells treated with RNAi in the presence of dox with the cell viability of cells treated with dox alone. Fold protection against dox was determined as the ratio between the mean cell viability of cells transfected with dsRNA in the presence of dox and cells treated with dox only.

Caspase activation assay

Kc cells were transfected in 96-well plates with the 62 dsRNAs that were confirmed in the primary screen followed by treatment with dox for 8 h to induce caspase activation. Each dsRNA was tested in triplicate and in at least two independent experiments. Caspase-3/7–like activity was quantified using a luciferin-labeled DEVD peptide substrate (Caspase-Glo 3/7 assay; Promega). Fold caspase-3/7–like activity was determined as the ratio between the mean caspase-3/7–like activity of cells transfected with dsRNA in the presence of dox and cells treated with dox only.

DIAP1 epistasis studies

Kc cells were transfected in 96-well plates with individual dsRNAs targeting the 62 genes confirmed in the initial screen. After a 24-h incubation, these cells were transfected with 1 μg diap1 dsRNA for an additional 48-h incubation when cell viability was quantified (as described in the Rescreen section). Fold protection against diap1 RNAi was determined as the ratio between the mean cell viability of cells transfected with the indicated dsRNA and diap1 RNAi and the mean cell viability of cells transfected with diap1 RNAi only. For the caspase activation assay, Kc cells were transfected with dsRNA as indicated followed by transfection with dsRNA against diap1 and an additional 24-h incubation.

Mammalian RNAi studies

Low-passage HeLa cells were transiently transfected (5 × 105/well in 96-well plates) with a pool of four siRNAs unless otherwise indicated (50 nM; predesigned ON-TARGETplus siRNAs; Dharmacon) using HiPerFect transfection reagent (QIAGEN). After a 48-h incubation, cells were treated with dox (concentrations and incubation times are noted in figures). siRNAs were tested in triplicate for each independent experiment. Cell viability and caspase-3/7 activity was quantified as described for Kc cells. Images were obtained using bright-field microscopy.

For detection of caspase cleavage, HeLa cells were transfected with siRNAs (1.5 × 105/well in six-well plates) followed by treatment with dox. Cells were lysed directly in SDS sample buffer and subjected to SDS-PAGE analysis. Caspases were detected by immunoblotting using the following antibodies: caspase-9 (R&D Systems), caspase-2 (Alexis), caspase-3 (Cell Signaling Technology), and cleaved caspase-8 (Cell Signaling Technology). hARD1 was detected using a rabbit polyclonal antibody (provided by...
M.C. Brahimi-Horn, University of Nice, Centre National de la Recherche Scientifique, Nice, France.

For hARD1 reconstitution experiments, HeLa cells were transfected with a pool of two siRNAs targeting the 5' untranslated region of hARD1 (sequences CGUACUGCGGCUUACGAAUU and GCCUAGCUGGCC-UUCAGCAU; Pharmacia) in six-well plates followed by a 24-h incubation. Transfection was performed by transient transfection reagent (Mirus) and an additional 24-h incubation. After dox treatment, cells were lysed and analyzed by Western blotting.

Microscopy

Images of HeLa cells were obtained using a microscope (Eclipse TE300; Nikon) with a 10× PH L objective lens and a camera (ORCAER; Hamamatsu) at room temperature. Openlab 3.1.7 acquisition software (Improvision) was used to analyze the images.

Online supplemental material

Fig. S1 shows a characterization of dox-induced cell death and controls for RNAi screen. Fig. S2 presents semi-quantitative analysis of dsRNAs to validate the RNAi library. Table S1 provides a comprehensive list of genes identified in the primary screen. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708090/DC1.

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