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Genome-Wide Silencing in *Drosophila* Captures Conserved Apoptotic Effectors

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Summary

Apoptosis is a conserved form of programmed cell death (PCD) firmly established in the etiology, pathogenesis and treatment of many human diseases. Central to the core machinery of apoptosis are the caspases and their proximal regulators. Current models for caspase control envision a balance of opposing elements, with variable contributions from positive regulators and negative regulators among different cell types and species¹. To advance a comprehensive view of components that support caspase-dependent cell death, we conducted a genome-wide silencing screen in the *Drosophila* model. Our strategy combined a library of dsRNAs together with a chemical antagonist of Inhibitor of Apoptosis Proteins (IAPs) that simulates the action of native regulators in the Reaper/Smac family². A highly validated set of targets necessary for death provoked by multiple stimuli was identified. Among these, *Tango7* is advanced here as a novel effector. Cells depleted for this gene resisted apoptosis at a step prior to induction of effector caspase activity and directed silencing of *Tango7* in the animal prevented caspase-dependent PCD. Unlike known apoptosis regulators in this model³, *Tango7* activity did not influence stimulus-dependent loss of *Drosophila* IAP1 (*DIAP1*) but, instead, regulated levels of the apical caspase *Dronc*. Likewise, the human *Tango7* counterpart, PCID1, similarly impinged on caspase 9, revealing a novel regulatory axis impacting the apoptosome.

Mammalian Smac proteins are thought to be functional orthologues of the *Drosophila* IAP antagonists referred to as RHG proteins (*Reaper*, *Head Involution Defective*, *Grim*, *Sickle*, *Jafrac*)³. Therefore, we tested the possibility that a small molecule Smac-mimetic (SM)⁴ might simulate the action of RHG proteins in *Drosophila* cells. Like RHG proteins⁵, the SM compound specifically bound DIAP1, a central brake against caspases³ (Fig. 1a). In *Drosophila* cell lines, the SM compound induced stereotypical apoptosis (Fig. 1b, supplementary movies), that was completely reversed either by peptide caspase inhibitors or RNAi depletion of the apical caspase *Dronc* (Fig. 1c), mirroring haemocytes lacking apoptosomal genes^{6,7}. Together, the findings establish the SM compound as a molecular mimetic of RHG proteins.

We conducted an RNAi-based genome scale screen to identify essential apoptotic determinants using the SM compound as a proximal apoptogenic stimulus (Fig. 2a, methods). Our platform used S2R+ cells and a library of dsRNAs targeting 13,071 annotated genes in *Drosophila*

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genome build 3 (Fig. 2a, methods) to capture targets that prevent SM-induced killing when silenced (Fig. 2c). Each dsRNA (supplemental Table 4) was tested in triplicate. Effects were typically reproducible (Fig. 2b) and assay quality, measured by Z-factor values, was consistently high⁸. Using both plate and position mean centering analyses (methods), we identified 42 candidate genes with protective activity meeting or exceeding a stringent z-score ≥ 3.1 threshold ($P < 0.001$). This collection includes the expected benchmark targets *Dronc*, *Dark* and the effector caspase *Drice* (Fig. 2c, Table 1, supplemental Table 1).

33 of the 42 candidate genes with a z-score greater than 3.1 were retested using a different dsRNA amplicon (Fig. 2d)⁹. To examine the biological relevance of this threshold, 42 targets conferring less potent activity ($2.1 < z < 3.1$) were also retested (supplemental Table 2). 19 candidates retested at least 3.1 standard deviations above the control mean, 16 of which had $z > 3.1$ in the original screen, while only 3 that survived this retest standard originally had $2.1 < z < 3.1$. Hence, our initial z threshold was experimentally relevant and predictive for reproducibility across different dsRNA amplicons.

We selected a set of 13 priority targets (Table 1) for further characterization based on conservation and/or implied links to human disease or development. For these, our validation strategy applied different assay methods, distinct apoptotic triggers and alternative non-overlapping dsRNAs¹⁰. We assessed new amplicons targeting each candidate by measuring effector caspase activity after UV or cycloheximide challenge, two well-established apoptotic stimuli¹¹. These data (Table 1) discriminate several target groups including: 1) genes that exert substantial activity across multiple stimuli (*Drice*, *Hrb27C*, *enok*, *Tango7*), 2) stimulus-dependent genes (*CG32626*, *CG7275*) and 3) genes with mild but consistently significant activity (*SNF4Agamma*, *eIF5*, *skt*).

Depletion of *Tango7* (*CG8309*) prevented killing induced by multiple apoptotic stimuli. Non-overlapping dsRNAs that target this gene (*Tango7*^{#1} and *Tango7*^{#2}) conferred protective activity comparable to benchmark amplicons that silence *Dronc* and *Dark* (Table 1, Fig. 3b). When tracked over a 22 hour time course following UV challenge, more than 95% of control cells initiated blebbing and died. However, like samples depleted for *Dronc*, cells silenced for *Tango7* were similarly prevented from apoptotic blebbing when directly visualized over this same period (Fig. 3a, supplementary movies). Together with DEVDase assays in Figure 3b, our results indicate that *Tango7* acts prior to effector caspase activation and prior to steps involved in apoptotic blebbing.

The combined results from multiple amplicons and three distinct assays validate *Tango7* as an apoptogenic effector in S2R+ cells. To test the relevance of *Tango7* during PCD *in vivo*, *Tango7* dsRNAs were targeted to the pupal retina where excessive interommatidial cells are eliminated through caspase-dependent cell death³. Two different insertions of the 8309R1 transgene (*Tango7R1* and *Tango7R3*) were compared to an irrelevant control dsRNA transgene (methods). Like retinas mutated for apical or effector caspases^{12,13}, retinas expressing either *Tango7R1* or *Tango7R3* retained significant numbers of extra interommatidial cells (Figure 3d–f). This effect was similar to animals lacking *drice*¹³ and was not seen with an irrelevant dsRNA strain from the same collection. Depletion of *Tango7* in the wing also prompted a range of defects which were consistent with failures in post-eclosion cell death and similar to canonical PCD mutants^{6,12,14,15} (supplemental Figure 1a–d). Low-penetrance phenotypes not shared with other PCD mutants suggest that *Tango7* may not be dedicated to apoptotic functions. Unlike canonical cell death mutants, occasional scars appeared in a minority of adult retinas silenced for *Tango7* (supplemental Figure 1e, f) and, hence, extra interommatidial cells could conceivably arise through activity unrelated to cell death. However, since scars are not evident in pupal stages, these infrequent defects manifest long after pruning of interommatidial cells and, more likely, they reflect pleiotropic roles for *Tango7* in cells that are not fated to die.

To investigate the mode of apoptosis regulation by *Tango7*, core determinants were investigated. Surprisingly, *Tango7* did not affect basal or stimulus-dependent loss of DIAP1 protein levels (Fig. 4a), a central point of control commonly observed during canonical apoptosis signaling in *Drosophila*³. Levels of the apoptosomal protein Dark were also unaffected (Fig. 4a). By contrast, silencing of *Tango7* caused a marked decline in pro-Dronc levels (Fig. 4b). This relationship was also observed in the Dark depleted state (Figure 4b) which causes pro-Dronc accumulation to unusually high steady state concentrations¹⁶. Therefore, under a wide dynamic range, *Tango7* positively regulates levels of this caspase. The findings also implicate a post-transcriptional mechanism linking *Tango7* activity to *Dronc* protein levels since, within the limits of our semi-quantitative rtPCR assay, the treatments had little or no effect on *Dronc* mRNA abundance (Fig. 3c).

To examine if the relationship between *Tango7* and *Dronc* (Fig 4b) reflects a conserved regulatory axis, we tested whether the human ortholog of *Tango7*, PCID1, might similarly impact caspase 9. Human T98G cells¹⁷ or IMR90^{E1A} cells¹⁸ were treated with gene-specific siRNAs and steady state levels of pro-caspase 9 were determined. As in *Drosophila*, a similar relationship between human counterparts was exposed in cell lines of distinct origins (Fig 4c). Here, PCID1 siRNAs caused a repression of pro-caspase 9 levels, with effects that were analogous to siRNAs that directly target caspase 9. To further explore links between PCID1 and apoptosis, siRNA-treated cells were challenged with an apoptogenic stimulus (UV) and tested for induction of effector caspase activity. As seen in Figure 4d, silencing either PCID1 or caspase 9 attenuated stimulus-dependent DEVDase. Furthermore, co-silencing of both targets reduced activity comparable to depletion of the pro-apoptotic effector, Bak¹⁹. Hence, in cells where levels of caspase 9 are rate-limiting, PCID1 is a relevant determinant of caspase activity. We also measured ATP in these experiments (not shown) and, by this criterion, neither caspase 9 siRNAs nor PCID1 siRNAs appeared cytoprotective. However, this result was anticipated since, unlike caspase-inhibited fly cells³, mammalian cells often die by alternate forms of cell death when caspase activity is blocked^{19,20}.

The highly validated gene set in Table 1 presents new opportunities for understanding how cells integrate death signals and execute apoptogenic responses. Like all RNAi screens, only rate-limiting targets were captured and *Drosophila* effectors not present in S2R⁺ cells were missed. Of note, *Dark*, *Dronc* and *Drice* dsRNAs were recovered among the top candidates, providing reassurance for the logistics of our platform. Targets recovered through our screen exposed previously unknown modalities that support apoptosis but the extent to which most of these generalize beyond the original *Drosophila* culture system is not known. In principle, even the effector featured here, *Tango7*, might regulate caspase-dependent cell death and *Dronc* in just S2R⁺ cells. However, this scenario is quite unlikely for at least two reasons. First, when *Tango7* is silenced in the retina, the exact same cells which persist are those which are known to die via caspase-dependent, canonical pathways. Second, our *Drosophila* studies successfully predicted cross-species apoptogenic activity for the counterpart of *Tango7* in human cells of distinct origin.

Tango7 depletion uniquely illustrates how cell survival can be sustained (despite stimulus-dependent loss of DIAP1, Fig. 4a), exposing a novel axis of regulation that bypasses a common apoptotic switch in flies and, instead, impacts the apoptosome³. We found no evidence for a physical association between *Tango7* and *Dronc* protein (not shown) and, despite less severe effects upon pro-Dronc levels, rescue by dsRNAs targeting *Tango7* were generally comparable to those targeting *Dronc*. Hence, *Tango7* could affect the post-translational processing or sub-cellular localization of pro-Dronc. Alternatively, *Tango7* could impact other apoptotic effectors, exerting effects beyond simply *Dronc* regulation. Consistent with these possibilities, *Tango7* was linked to golgi activity²¹ but, since genes needed for general golgi functions were not recovered here, links between *Tango7* and *Dronc* evidently do not reflect compromised

golgi. Whatever the mechanism, this regulatory axis appears to be conserved since the *Tango7* counterpart, PCID1, similarly regulated caspase 9 levels and DEVDase in human cells (Fig 4C). In a recent study of pancreatic cancers²², PCID1 expression was repressed at least six-fold in all five patient tumors profiled. Furthermore, PCID1 was also implicated as a determinant of Herpes Simplex Virus susceptibility²³. It will be important to examine whether these possible links to human disease reflect apoptosis related activity or, perhaps, non-apoptotic functions reported for caspase 9²⁴.

METHODS SUMMARY

RNAi screen and validation

18,000 cells in serum/antibiotic-free Schneider's media were seeded in each well containing 0.5µg dsRNA (Ambion Silencer RNAi library or synthesized dsRNA) and incubated for 1 hour before adding 100µl serum-supplemented media. 3 days later, the media was replaced with SM-containing media. Cell viability was assayed 2 days later using CellTiter-Glo (Promega) in a plate reader (Envision multimode). Every assay plate included 4 control wells containing *Dronc* dsRNA. Of 429 sample plates assayed in the screen, 76% (325 plates) had excellent assay quality ($Z \geq 0.5$) and only 2% were unacceptable ($Z < 0$). The Z-factor for each plate⁸ and plate mean centered z-score for each well (n=92 sample wells) were calculated. To correct for systematic bias/edge effects²⁵, the position mean centered z-score for each well is calculated by its plate mean centered z-score minus the position average, divided by the position standard deviation (n=143 plates per triplicate). Genes with $z > 3.1$ ($P < 0.001$) from either plate mean or position mean centered normalizations were considered together as primary candidates. For secondary screens and subsequent RNAi experiments, dsRNA synthesis and treatment was as described^{9,26} (Supplementary Table 3). Briefly, cells were cultured 72 hours to deplete target gene product before analysis. Effector caspase (DEVDase) activity was measured using Caspase3/7-Glo (Promega) 6 hours after apoptosis stimulation.

METHODS

Fly stocks

Tango7R1 denotes the GAL4-responsive the RNAi line targeting CG8309 with a genotype $w; P\{UAS-8309R-1\}2$ on the 2nd chromosome (NIG, Japan). This transgene was mobilized by standard methods and an insertion, designated *TangoR3*, was recovered on the 3rd chromosome. *3523R2* denotes the GAL4-responsive *CG3523* RNAi line $w; P\{UAS-3523R-2\}3$ (stock#3523R-2, NIG). *5887R1* denotes the GAL4-responsive *CG5887* RNAi line $w; P\{UAS-5887R-1\}$ (stock#5887R-1, NIG). For pupal eye analyses, genotypes for *Tango7* are *UAS-dicer2/+; gmr-GAL4/UAS-Tango7R1*, or *UAS-dicer2/+; gmr-GAL4/+; UAS-Tango7R3/+*; or, as control, *UAS-dicer2/+; gmr-GAL4/UAS-5887R1*. The 3 independent wing-specific *vestigial-GAL4* driver lines, *vg-GAL4#1* denotes $y^1 w^{1118}; P\{w^{+mC}=vgM-GAL4.Exel\}3$ (stock#8223, Bloomington), *vg-GAL4#2* denotes $P\{w^{+mC}=vgMQ-GAL4.Exel\}1, y^1 w^{1118}$ (stock#8231, Bloomington) and *vg-GAL4#3* denotes $y w hsp70-flp; P\{mini w^+, GAL4\}vgBE P\{mini w^+, UAS-flp\}; P\{y^+, hsp70-cd2\} P\{mini w^+, FRT\}79 P\{ry^+, hsp70-neo, FRT\}82 P\{mini w^+, hsp70-HA-gfp, smo^+\}$.

Cell culture, UV challenge and western analyses

S2R+ cells were cultured in Schneider's media with 10% Fetal Bovine Serum, 25U/mL penicillin, 25µg/mL streptomycin at 25°C (all cell culture reagents from Invitrogen except FBS, Atlas Biologicals). Unless otherwise indicated, media in all procedures refer to the above. T98G and IMR90^{E1A} cells were cultured in DMEM supplemented with 10% FBS. For western analysis, samples were lysed in buffer A with 1% TX-100. Blots were probed with 1:1000 α-DIAP1 (gift from Kristin White), 1:1000 α-Dronc (gift from Bruce Hay), 1:1000 α-Dark⁷,

1:5000 α -tubulin (E7, Developmental Studies Hybridoma Bank, University of Iowa). *Drosophila* cells treated with two dsRNAs were exposed to 15 μ g/ml of each indicated dsRNA, for a total dsRNA concentration of 30 μ g/ml. AmpRx2 denotes cells exposed to 30 μ g/ml of control AmpR dsRNA. Cells were analyzed either untreated or 6 hours after UV challenge (100 mJ/cm²). Relative changes in band intensity were obtained by comparing integrated densitometric values (IDV). Human cells were harvested five days after siRNA transfection, and 10 μ g total protein were separated by 10% SDS-PAGE. Caspase-9 and PCID1 levels were analyzed using anti-caspase 9 (Cell Signaling) and α -PCID1 (Proteintech Group, Inc.).

Smac-mimetic binding

Cells were lysed in 20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol (buffer A), 1% Triton-X100 buffer with protease inhibitors (#1836170 Roche). Biotinylated-SM or Biotin was incubated with avidin-conjugated beads and 10mg/ml BSA in PBS for 4 hours at 4 °C. The beads were then incubated with precleared lysate (~1–2 mg protein) overnight before analysis.

Primary and secondary RNAi Screen

The primary screen was optimized for S2R+ cells and conducted using a 'direct soaking' protocol, together with the Silencer *Drosophila* RNAi library (Ambion/Applied Biosystems) targeting 13,071 genes from *Drosophila* genome build 3. Sequences for all dsRNAs in the library are available in Table 4 of supplemental materials. The RNAi library or synthesized dsRNAs were plated in 96-well microplates (Corning) using Beckman FX liquid handlers. Every assay plate included 4 control wells containing *Dronc* dsRNA. Each well contained 0.5 μ g dsRNA in 20 μ l of SF900 media. 18,000 cells in 30 μ l serum/antibiotic-free Schneider's media were seeded in each well and incubated for 1 hour before adding 100 μ l media. 3 days later, the media was replaced with 80 μ l 2.5 μ M SM media. Cell viability was assayed 2 days later using CellTiter-Glo (Promega) in a plate reader (Envision multimode). The plate mean centered z -score for each well is its luminescence value minus the plate average, divided by the plate standard deviation ($n=92$ sample wells). The Z-factor for each plate was calculated as described⁸. Low quality ($Z<0$) plates were excluded from subsequent secondary retests and analyses. To correct for systematic bias/edge effects²⁵, the position mean centered z -score for each well is calculated by its plate mean centered z -score minus the position average, divided by the position standard deviation ($n=143$ plates per triplicate). Average position mean centered z -score for each amplicon is calculated from the individual position mean centered z -scores of the triplicates. Genes with $z>3.1$ from either plate mean or position mean centered normalizations were considered together as primary candidates. For secondary screens and subsequent RNAi experiments, dsRNAs synthesis and treatment was as described^{9,26} (Supplementary Table 3). Cells were cultured 72 hours to deplete target gene product before analysis. Effector caspase (DEVDase) activity was measured using Caspase3/7-Glo (Promega) 6 hours after apoptosis stimulation according to manufacturer's instructions.

Time-lapse microscopy

Cells pretreated with dsRNAs were tracked by time-lapse microscopy for 22 hrs after UV exposure (90mJ/cm² UVC). Photomicrographs were captured at 6-minute intervals on a Zeiss Axiovert 200M inverted microscope equipped with a Marzhauser programmable stage, Nikon DXM1200F camera and controlled by Metamorph software (Molecular Devices). Movie images were assembled and analysed using ImageJ (NIH). At least 100 cells were tracked in each treatment. Dead cells are scored by morphology of rounding up, blebbing or fragmentation into corpses.

Pupal eye analyses

White prepupae were isolated at 0hr and aged 46–48hr. at 25°C. To outline cell borders, pupal eyes were dissected and stained with mouse α -DiscsLarge (Developmental Studies Hybridoma Bank, 1:1000) and α -mouse Fluorecein (Vector Laboratories, 1:250) as described in^{6,14}. Interommatidial cell counts were modified from²⁷. Hexagonal units connecting six 'ommatidia centers' that completely surround one ommatidium were counted. Secondary or tertiary pigment cells inside or partly inside hexagonal assemblies were counted as a single cell.

siRNA Transfection

siRNAs to CASP9 and PCID1 were ON-TARGETplus SMARTpool siRNA, and the control siRNA used was ON-TARGETplus Non-targeting pool (Dharmacon). T98G and IMR90^{E1A} cells were transfected with 64 nM siRNAs targeting the genes indicated using Dharmafect Duo (Dharmacon) and Optimem according to manufacturer's instructions. Pools contain 4 siRNAs for each indicated gene. Five days later, transfected cells were challenged with UV (100 mJ/cm²). Effector caspase (DEVDase) activity was measured using Caspase3/7-Glo (Promega) 18 hours after UV stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. A smac-mimetic (SM) compound exerts broad cross-species IAP antagonist activity

a, Cell lysates were incubated with Biotinylated-SM, or biotin. Pellets (P) of avidin-conjugated beads and supernatants (S) were probed with α -DIAP1 or α -tubulin.

b, SM induces apoptotic blebbing in S2R+ cells within 6hr. of treatment (supplementary movies) shown here 24hr. SM triggers similar effects in other fly cell lines and *ex vivo* primary cells ^{6,7}.

c, After treatments indicated, viability was measured using CellTiter-Glo (ALU denotes arbitrary luminescence units, error bars $\pm$ SD, n=3 sample wells).

Figure 2. A genome-wide screen captures apoptogenic effectors

a, Screen schematic

b, Plate mean centered z-scores for triplicate samples of each dsRNA (t1, t2 and t3) plotted against each other (r^2 denotes correlation coefficient).

c, Average position mean centered z-score plotted in rank format. Arrowheads highlight 'landmark genes' with significant pro- and anti-apoptotic activity. Dotted line is $z=3.1$.

d, Schematic for validation of candidates.

Figure 3. *Tango7* is an effector of apoptosis

a, Time-lapse microscopy of dsRNA treated cells after UV exposure (see supplementary movies) and cumulative counts (y-axis) of live (blue) or dead (red) cells. Similar results were observed with *Tango7*^{#1} dsRNA (here) or *Tango7*^{#2} dsRNAs (not shown).

b, DEVDase activity 6 hours after UV irradiation in RNAi depleted cells. *Tango7*^{#1} and *Tango7*^{#2} are non-overlapping dsRNA amplicons targeting the *Tango7* transcript (see methods, ALU denotes arbitrary luminescence units, error bars $\pm$ SD, n=3 different sample wells).

c, Semi-quantitative rtPCR detects relative transcript levels of *Tango7*, *Dronc* and control, *RP49*, in dsRNA treated cells as in (a).

d, e and **f** examine caspase-dependent cell death in the pupal eye. Transgenic dsRNAs silencing *Tango7* **e**, or an irrelevant control (5887R1) **d**, were targeted using the gmr-GAL4 eye 'driver' (see ^{12,13} and methods). Extra interommatidial cells shown (**e**, outlined red) are from *Tango7R1*. **f**, in control retinas, a majority (63%) of hexagonal assemblies had stereotypical numbers of interommatidial cells (15). Less than 5% showed ≥ 17 cells per ommatidia (N=87). *Tango7R1* produced 28% of ommatidia with ≥ 17 cells (N=118) and a minority (40%) showed stereotypical numbers. *Tango7R3* produced 77% of ommatidia bearing ≥ 16 cells (N=62). Differences between control and *Tango7* are significant by two-tailed unpaired t-test (*Tango7R1* p=0.0002; *Tango7R3* p<0.0001). Bars under x axis, **f**, indicate values for dark and *dronc*²⁸ (solid) or *drice* (open)¹³.

Figure 4. Tango7 and its human ortholog regulate apoptosomal caspases

a, S2R+ cells treated with indicated dsRNAs were analyzed untreated (-) or after UV challenge (+). The same blot was probed with α -Dark and α -DIAP1. α -DIAP1 detects full length and modified forms (open arrowheads)²⁹. α -Dark detects full length and cleaved Dark proteins (open arrowheads)⁷. Non-specific, α -DIAP1 band (*) is loading control.

b, S2R+ cells treated with indicated dsRNAs were probed with α -Dronc. Non-specific band (*) is loading control. In 3 independent experiments, compared to AmpRx2 control, relative reductions in pro-Dronc band intensity for Tango7^{#1}+AmpR and Tango7^{#2}+AmpR ranged 63–86% and 53–85% respectively. Compared to Dark+AmpR treated cells, relative reductions in

band intensity for co-silencing Tango7^{#1}+Dark and Tango7^{#2}+Dark ranged 36–61% and 33–54% respectively.

c, Human T98G cells or human IMR90^{E1A} cells were treated with siRNAs targeting PCID1 (PCI), Caspase-9 (C9), or a non-target control (C). Procaspace 9 (FL C9), cleaved caspase-9 (arrowhead) and PCID1 (PCI) were detected. Loading control (*) non-specific band. Two independent trials with the T98G line, T98G_(t1) and T98G_(t2) are shown.

d, IMR90^{E1A} cells transfected with siRNAs targeting PCID1 (PCI), Caspase 9 (C9), Bak or Bax were subsequently UV challenged and assayed for DEVDase activity. C9/PCI samples received siRNAs targeting both caspase 9 and PCID1. Triplicate data was normalized against cells transfected with non-targeting control siRNAs (C) treated in parallel. Error bars show SD. Significance at $p < 0.05$ * or $p < 0.01$ ** determined by student's t-test (2 tailed). Three independent trials gave similar results. C9 or PCI siRNAs had negligible effects on basal DEVDase since, absent UV challenge, DEVDase activity varied an average of 2.3% (C9 siRNA) or 1.6% (PCI siRNA) relative to control.

Characterization of High Rank Candidate Genes

Table 1

% Caspase Inhibition ¹ (t-Test ²) Gene (Human)	Description	UV		Cycloheximide	
		amplicon 1	amplicon 2	amplicon 1	amplicon 2
Control					
Ampr	Bacteria gene	0 ± 3.3		0 ± 3.5	
Known Benchmarks					
Drone (CASP9 ³)	Apical caspase	63.5 ± 4.9 (P=1.6E-10)		71.4 ± 1.8 (P=7.6E-13)	
Dark (APAF1 ³)	Caspase activation adaptor	55 ± 7.8 (P=1.9E-08)		47 ± 19.8 (P=0.00019)	
Candidates					
Drice (CASP3 ³)	Effector caspase	59 ± 7.5 (P=7.3E-09)	30.1 ± 8.5 (P=1.1E-05)	67.2 ± 12.4 (P=1.6E-07)	48.9 ± 24.2 (P=0.00063)
Hrb27C (DAZAPI1)	Ribonucleoprotein	59.9 ± 6.5 (P=2.0E-09)	54.7 ± 4.5 (P=3.7E-10)	40.4 ± 16.8 (P=0.00019)	27.1 ± 29.5 (P=0.049)
enok (MYST3)	Histone acetyltransferase	57.7 ± 5.4 (P=7.6E-10)	38.1 ± 4.4 (P=1.2E-08)	47.6 ± 14.7 (P=1.6E-05)	24.1 ± 13.1 (P=0.0014)
SNF4Agamma (PRKAG1)	SNF4/AMP-activated kinase	12 ± 10 (P=0.019)	22.1 ± 3.5 (P=5.2E-07)	8.7 ± 6.3 (P=0.015)	17 ± 4.3 (P=2.1E-05)
CG32626 (AMPD2)	AMP deaminase	18.3 ± 4.4 (P=1.0E-05)	14.9 ± 2.4 (P=4.8E-06)	7.4 ± 3.9 (P=0.0062)	2.6 ± 7.6 (P=0.46)
CG40084 (GNM2)	Cystathionine beta-synthase domain	9.6 ± 9.2 (P=0.037)	9.9 ± 2.9 (P=0.00026)	5.4 ± 3.3 (P=0.020)	6.1 ± 10.1 (P=0.19)
ATPalpha ⁴ (ATPIA3)	Na ⁺ ,K ⁺ ATPase alpha subunit	22.1 ± 3.9 (P=1.0E-06)	-12.9 ± 28.8 (P=0.30)	4.8 ± 11.3 (P=0.34)	-2.7 ± 2.8 (P=0.17)
CG7275 (WDSOF1)	WD40, Sof1 domains	27.9 ± 1.2 (P=3.0E-09)	30.9 ± 4 (P=5.0E-08)	9.5 ± 7.6 (P=0.020)	14.4 ± 7 (P=0.0011)
Tango7 (PCID1)	PCI domain	66 ± 7.7 (P=3.2E-09)	68 ± 7.4 (P=1.7E-09)	58.4 ± 9.9 (P=8.6E-08)	60.9 ± 10.7 (P=1.1E-07)
Myb (MYBL2 ³)	Myb oncogene like transcription factor	16.4 ± 4.3 (P=2.5E-05)	20.8 ± 3.1 (P=5.4E-07)	12 ± 9.8 (P=0.018)	10.8 ± 17.6 (P=0.17)
eIF5 (EIF5)	Translation initiation factor	28.4 ± 5.9 (P=1.2E-06)	13.1 ± 2.3 (P=1.3E-05)	19 ± 2.9 (P=1.2E-06)	9.7 ± 9 (P=0.033)
Ikbl (STK11 ³)	Serine/threonine kinase	5.7 ± 5.4 (P=0.051)	36.7 ± 11.2 (P=1.7E-05)	3.4 ± 4 (P=0.15)	10.6 ± 22.1 (P=0.27)
skt1 (PIP5K1A ³)	PIP5-kinase	16.1 ± 3.8 (P=1.4E-05)	19.4 ± 3.2 (P=1.3E-06)	17.7 ± 4.6 (P=2.1E-05)	16.2 ± 9.3 (P=0.0025)

¹ Caspase activity measured 6 hours after treatment with 90 mJ/cm² UVC or 250 μM cycloheximide. Percent caspase activity was calculated relative to control mean (100%) for each experiment. Data shown are mean percent inhibition ± S.D. from 2 independent experiments (n=3 wells for each experiment).

² P-values for 2-tail t-tests were calculated by comparing percent inhibition of each gene with the control for each treatment. High consistency and reproducibility across amplicons indicate requisite functions in apoptosis.

³ Human genes involved in cell death/survival pathways.

⁴ Only amplicon 1 significantly affects UV-stimulated caspase activation. The specificity of amplicon 1 or ATPalpha to rescuing ATP viability and caspase activation has not been tested.