Steroid Hormone Control of Cell Death and Cell Survival: Molecular Insights Using RNAi

Suganthi Chittaranjan¹, Melissa McConechy¹, Ying-Chen Claire Hou¹, J. Douglas Freeman¹, Lindsay DeVorkin¹, Sharon M. Gorski¹,²*

¹The Genome Sciences Centre, BC Cancer Agency, Vancouver, British Columbia, Canada, ²Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, British Columbia, Canada

Abstract

The insect steroid hormone ecdysone triggers programmed cell death of obsolete larval tissues during metamorphosis and provides a model system for understanding steroid hormone control of cell death and cell survival. Previous genome-wide expression studies of Drosophila larval salivary glands resulted in the identification of many genes associated with ecdysone-induced cell death and cell survival, but functional verification was lacking. In this study, we test functionally 460 of these genes using RNA interference in ecdysone-treated Drosophila (2)mbn cells. Cell viability, cell morphology, cell proliferation, and apoptosis assays confirmed the effects of known genes and additionally resulted in the identification of six new pro-death related genes, including sorting nexin-like gene SH3PX1 and Sox box protein Sox14, and 18 new pro-survival genes. Identified genes were further characterized to determine their ecdysone dependency and potential function in cell death regulation. We found that the pro-survival function of five genes (Ras85D, Cp1, CG13784, CG32016, and CG33087), was dependent on ecdysone signaling. The TUNEL assay revealed an additional two genes (Kap-x3 and Smr) with an ecdysone-dependent cell survival function that was associated with reduced cell death. In vitro, Sox14 RNAi reduced the percentage of TUNEL-positive (2)mbn cells (p<0.05) following ecdysone treatment, and Sox14 overexpression was sufficient to induce apoptosis. In vivo analyses of Sox14 RNAi animals revealed multiple phenotypes characteristic of aberrant or reduced ecdysone signaling, including defects in larval midgut and salivary gland destruction. These studies identify Sox14 as a positive regulator of ecdysone-mediated cell death and provide new insights into the molecular mechanisms underlying the ecdysone signaling network governing cell death and cell survival.

Introduction

Steroid hormones are small hydrophobic signaling molecules which bind to their receptors to control gene expression and initiate the regulation of growth, development, homeostasis and programmed cell death (PCD) [1]. Components of the steroid-regulated PCD transcriptional regulatory cascades in insects and mammals have been well characterized. For example, in vertebrates, steroid hormone glucocorticoids regulate the removal of excess thymocytes during T-cell maturation [2,3]. In insects, the transcriptional cascade induced by the steroid hormone 20-hydroxycyclodecylone (ecdysone) has been implicated in the activation of PCD in larval intersegmental muscle [4–6], newly eclosed adult central nervous system [7,8], larval salivary glands [9], and larval midgut [10]. Deregelation of the hormonal control of PCD in humans has been associated with various pathological conditions, including cancer and the degenerative disorder Alzheimer’s Disease [1,11,12]. Given the functional conservation of many genes in humans and Drosophila, experiments to identify the genes required for hormonal control of Drosophila PCD will provide not only a better molecular understanding of the process itself, but may also be valuable in the context of human disease treatment and diagnostics.

During metamorphosis of Drosophila, two stage-specific sequential pulses of ecdysone activate first the transformation of larvae into pupae, and then the transformation of pupae into adult flies. The ecdysone pulses regulate the destruction of obsolete larval tissues, and the differentiation and morphogenesis of adult tissues which arise from small clusters of progenitor cells, [7,8,13–16]. The first ecdysone pulse occurs at the late third instar larval stage and triggers puparium formation. In addition, the larval midgut undergoes histolysis and the future adult midgut tissue envelopes it by 2 hrs after puparium formation (APF) [9,10]. A second ecdysone pulse occurs 10 hrs APF and triggers the death of larval salivary glands [9]. Previous studies indicated that larval midguts and salivary glands employ similar, yet distinct, genetic mechanisms during steroid induced programmed cell death [10].

Several studies have identified some of the components involved in the transcriptional cascade upstream of PCD of salivary glands in Drosophila. The ecdysone receptor is a heterodimer of the nuclear receptors ecdysone receptor (EcR) and ultraspiracle (USP) [17]. The heterodimer complex binds to the steroid hormone ecdysone and induces the transcription of the early genes E93 (DNA binding protein), BR-C (zinc finger transcription factor), E74 (ETS-domain transcription factor), and E75 (orphan nuclear receptor) [13,18–22]. The EcR:USP complex and E93, BR-C and E74 proteins in turn activate transcription of several pro-death genes including reaper (rpr), head involution defective (hid) and grim which function similarly to mammalian SMAC/DIABLO, the APAF-1

Citation: Chittaranjan S, McConechy M, Hou Y-CC, Freeman JD, DeVorkin L, et al. (2009) Steroid Hormone Control of Cell Death and Cell Survival: Molecular Insights Using RNAi. PLoS Genet 5(2): e1000379. doi:10.1371/journal.pgen.1000379

Editor: Eric Rulifson, University of California San Francisco, United States of America

Received January 25, 2008; Accepted January 12, 2009; Published February 13, 2009

Copyright: © 2009 Chittaranjan et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by The British Columbia Cancer Foundation and by CIHR grant MOP-78882 to SMG.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: sgorski@bcgsc.ca
Author Summary

Hormones regulate complex signaling pathways required for the differentiation, growth, survival, and death of cells in diverse organisms. The insect steroid hormone 20-hydroxy ecdysone (ecdysone) triggers cell death of oblate larval tissues, such as the midgut and salivary glands, during metamorphosis and provides a model system for understanding steroid hormone control of cell death and cell survival. Previous studies identified many genes and proteins associated with fruit fly salivary gland cell death, but functional verification was lacking. Here, we have analyzed 460 of those genes using RNAi, a genetic approach to inhibit gene function, to assess their possible cell death or cell survival related function. To our knowledge, this is the first large-scale functional screen for genes involved in steroid hormone regulated cell death and cell survival. We identified several novel ecdysone regulated components with a cell death/survival role, including genes with no previously known function. *In vivo* analyses of animals harboring an RNAi construct targeting the transcription factor *Sox14*, one of the genes identified, confirmed its role as a positive regulator of ecdysone-mediated cell death. Our results provide a foundation for further studies of the molecular mechanisms by which steroid hormones control the death and survival of cells.

Hormones regulate complex signaling pathways required for the differentiation, growth, survival, and death of cells in diverse organisms. The insect steroid hormone 20-hydroxy ecdysone (ecdysone) triggers cell death of oblate larval tissues, such as the midgut and salivary glands, during metamorphosis and provides a model system for understanding steroid hormone control of cell death and cell survival. Previous studies identified many genes and proteins associated with fruit fly salivary gland cell death, but functional verification was lacking. Here, we have analyzed 460 of those genes using RNAi, a genetic approach to inhibit gene function, to assess their possible cell death or cell survival related function. To our knowledge, this is the first large-scale functional screen for genes involved in steroid hormone regulated cell death and cell survival. We identified several novel ecdysone regulated components with a cell death/survival role, including genes with no previously known function. *In vivo* analyses of animals harboring an RNAi construct targeting the transcription factor *Sox14*, one of the genes identified, confirmed its role as a positive regulator of ecdysone-mediated cell death. Our results provide a foundation for further studies of the molecular mechanisms by which steroid hormones control the death and survival of cells.

Characterization of Ecdysone-Induced l(2)mbn Cell Death

To validate our experimental system, we conducted cell viability, cell death, transcription and RNAi assays in ecdysone-treated l(2)mbn cells using known ecdysone signaling and apoptosis genes. First, to verify previous findings [42–44] of ecdysone treatment effects on *Drosophila* l(2)mbn cells, we employed multiple assays over a time course of ecdysone treatment. To assess cell viability, we used the trypan blue exclusion [55] and 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate (WST-1) based cell viability (Roche Diagnostics) assays. Both assays indicated that the majority of cells are non-viable by 72 hours following treatment with 10 μM ecdysone (Figure 1A and 1D). To specifically measure cell death, nuclei were stained with DAPI and the percent TUNEL positive cells, respectively, indicating that the reduced cell viability is due, at least in part, to increased cell death. In addition, we used electron microscopy (EM) to examine morphological features of l(2)mbn cells following ecdysone treatment. Consistent with previous reports [42], we observed features representative of apoptosis, autophagy and phagocytosis in the ecdysone treated cells (data not shown).

To determine the expression profile of representative ecdysone regulated transcription factors and apoptosis genes in l(2)mbn cells, we employed quantitative reverse transcription PCR (QRT-PCR) and measured transcript levels following 24, 48 and 72 hrs ecdysone treatment. Since we observed features of autophagy after ecdysone treatment, we also quantitated the expression levels of several autophagy genes to determine if their expression was ecdysone regulated in our experimental system. Our QRT-PCR results (Figure 1B) indicate that the early transcription factors *Br-C* and *E75* had the relatively highest expression levels at 24 hrs (10 and 13 fold increase in expression, respectively, compared to untreated control cells) and then decreased after 48–72 hours (at 48 hours, 2 and 7.9 fold increase in expression, respectively, compared to control cells). As demonstrated in Figure 1B, *E93*, *raper*, *drone* and *hid* demonstrated elevated expression levels by 24 hrs (58, 5.8, 3.4, and 2.3 fold increase respectively) which remained elevated or continued to increase at 48 and 72 hours (at 48 hours, 126, 36, 4.4 and 4.7 fold increase in expression, respectively). These observations suggest that the transcriptional cascade for the representative ecdysone signaling and apoptosis genes is similar between ecdysone-treated l(2)mbn cells and dying *Drosophila* larval salivary glands. Although we detected expression of autophagy genes in l(2)mbn cells, we observed no significant differential expression compared to untreated cells (i.e. below the arbitrarily chosen 2 fold cut-off level) (Figure 1B) up to 72 hrs following ecdysone treatment, indicating that the autophagy genes tested are not transcriptionally regulated in this system at these timepoints (Figure 1B).

To test the sensitivity of our RNAi strategy, we treated l(2)mbn cells with dsRNA corresponding to representative ecdysone signaling (*Ecr*, *BR-C* and *E75*) and apoptosis (*drone*, *rpr*, *hid* and *diap1*) related genes. First, to determine the knock-down efficiency of RNAi for the genes described above, we measured their expression levels at 72 hrs by QRT-PCR in ecdysone-treated cells
with or without dsRNA. For all the genes tested, the transcript knock-down ranged between 62–90% (for examples, see Figure 1C). Next, the WST-1 assay was used to measure cell viability following RNAi and ecdysone treatment. We found that treatment of l(2)mbn cells with ecdysone and dsRNAs corresponding to EcR, BR-C, rpr, hid and dronc had elevated levels of expression (at least 4 fold increase) in ecdysone treated (10 μM) l(2)mbn cells relative to untreated control l(2)mbn cells. The dotted lines above the bars for E93 indicate that the fold change in expression exceeded the existing scale (48 hrs, 126 fold change; 72 hrs, 371 fold change). The autophagy genes, DmAtg4-like (CG6194), DmAtg6 (CG5429), and DmAtg5 (CG1643), did not show differential expression following ecdysone treatment. (C) QRT-PCR analysis of gene transcripts following treatment with the indicated dsRNAs and ecdysone. As shown here, the knockdown ranged between 63% and 90% for the representative gene transcripts tested following 72 hr dsRNA and ecdysone treatment compared to ecdysone treatment alone. (D) Cells treated with dsRNA corresponding to BR-C, EcR, dronc, rpr, and hid showed significantly (p≤0.05) increased levels of cell viability and those treated with dsRNA corresponding to diap-1 showed significantly (p≤0.05) reduced levels of cell viability compared to cells treated with ecdysone and human dsRNA NM_138278 (negative control). Cell viability was measured by the WST-1 assay (A450–A650). The dotted lines above the bar for EcR indicate the A450–A650 value (= 1.0) exceeded the existing scale. For (A)–(D), the error bars represent the SD of triplicate samples.

doi:10.1371/journal.pgen.1000379.g001

**Figure 1.** Ecdysone signaling and apoptosis genes are differentially expressed and required for cell death in ecdysone treated l(2)mbn cells. (A) l(2)mbn cells treated with 10 μM ecdysone showed a >70% reduction in live cells after 72 hours. Cells were exposed to 10 μM ecdysone for the times indicated. Surviving cells were counted by Trypan blue exclusion and percent live cells were calculated by comparing ecdysone-treated cells with untreated cells (100%). (B) QRT-PCR expression profiling showed that the ecdysone induced genes E75, BR-C, E93, rpr, hid and dronc had elevated levels of expression (at least 4 fold increase) in ecdysone treated (10 μM) l(2)mbn cells relative to untreated control l(2)mbn cells. The dotted lines above the bars for E93 indicate that the fold change in expression exceeded the existing scale (48 hrs, 126 fold change; 72 hrs, 371 fold change). The autophagy genes, DmAtg4-like (CG6194), DmAtg6 (CG5429), and DmAtg5 (CG1643), did not show differential expression following ecdysone treatment. (C) QRT-PCR analysis of gene transcripts following treatment with the indicated dsRNAs and ecdysone. As shown here, the knockdown ranged between 63% and 90% for the representative gene transcripts tested following 72 hr dsRNA and ecdysone treatment compared to ecdysone treatment alone. (D) Cells treated with dsRNA corresponding to BR-C, EcR, dronc, rpr, and hid showed significantly (p≤0.05) increased levels of cell viability and those treated with dsRNA corresponding to diap-1 showed significantly (p≤0.05) reduced levels of cell viability compared to cells treated with ecdysone and human dsRNA NM_138278 (negative control). Cell viability was measured by the WST-1 assay (A450–A650). The dotted lines above the bar for EcR indicate the A450–A650 value (= 1.0) exceeded the existing scale. For (A)–(D), the error bars represent the SD of triplicate samples.

doi:10.1371/journal.pgen.1000379.g001

with or without dsRNA. For all the genes tested, the transcript knock-down ranged between 62–90% (for examples, see Figure 1C). Next, the WST-1 assay was used to measure cell viability following RNAi and ecdysone treatment. We found that treatment of l(2)mbn cells with ecdysone and dsRNAs corresponding to EcR, BR-C, rpr and hid resulted in increased cell viability (p<0.05) compared to cells treated with a negative control, a human dsRNA NM_138278 [56] (Figure 1D; Table 1). Treatment of l(2)mbn cells with dsRNA corresponding to either E75B (Table 1) or diap-1 (Figure 1D; Table 1) decreased cell viability significantly (p<0.001) as assayed by WST-1. We confirmed that the change in viability of the l(2)mbn cells treated with ecdysone and RNAi was due to alterations in cell death by employing the TUNEL and DAPI assay for selected genes (Table 2).

**RNAi Screen Identifies Novel Genes that Affect Cell Survival and Cell Death**

To identify additional genes that function in ecdysone-mediated cell death or cell survival, we conducted an RNAi screen (Figure 2).
Based on genome-wide transcript and protein expression studies conducted previously in *Drosophila* larval salivary glands [23,24,40], there are a large number of genes and proteins that could affect ecdysone-mediated PCD but have not been tested functionally. Here, we conducted a systematic study of 460 of these genes which included all of the annotated genes from our previous study [24] that showed a significant (p<0.05 and 5 fold difference) increase or decrease in expression levels in salivary glands immediately prior to PCD. The WST-1 assay was used as a primary screen to assess effects of the 460 dsRNAs on cell viability (Table S1). Using this assay, we identified five genes reported already to have a pro-survival role based on a previous RNAi screen [47,49,57]; (Table 1 and Table S1). In addition, we identified and validated another 20 genes with corresponding dsRNAs that significantly increased or decreased cell viability (Table 1). All of these 20 genes were validated with completely non-overlapping dsRNAs. In total, our final gene set for further analyses consisted of 18 genes with corresponding dsRNAs that

### Table 1. Candidate pro-death and pro-survival genes identified by RNAi and cell viability assay.

| Symbol or CG number of gene targeted | WST-1 assay: dsRNA-ecdysone | WST-1 assay: dsRNA only | Ecdysone dependency | predicted/known function | HGNC Symbol: Ortholog in human (mouse) |
|--------------------------------------|-----------------------------|------------------------|---------------------|--------------------------|--------------------------------------|
| **Control pro-death genes**          |                             |                        |                     |                          |                                      |
| EcR                                  | 7E-05                       | 2E-02                  | dependent           | ecdysone receptor, transcription factor | NR1H3                                |
| Hid                                  | 3E-03                       | 1E-01                  | dependent           | apoptosis                |                                      |
| BR-C                                 | 2E-02                       | 3E-01                  | dependent           | transcription factor activity | ZBT12                                |
| dronc (Nc)                           | 2E-02                       | 4E-01                  | dependent           | caspase activity         |                                      |
| **Ecdysone dependent candidate pro-death genes** |                             |                        |                     |                          |                                      |
| Rpl13A a                             | 6E-05                       | 2E-03                  | dependent           | 60S ribosomal protein L13a | XR_000922.1                          |
| Sox14                                | 7E-04                       | 1E-01                  | dependent           | transcription factor     | Sox 4,11,22                          |
| Rp56 b                               | 2E-03                       | 3E-02                  | dependent           | 40S ribosomal protein S6 | RPS6                                  |
| RpLP1 a                              | 3E-03                       | 3E-03                  | dependent           | 60S acidic ribosomal protein P1 | RPLP1                                |
| Rp55                                 | 6E-03                       | 2E-03                  | dependent           | 40S ribosomal protein S5 | RPS5                                  |
| SH3PX1 b                             | 2E-02                       | 5E-03                  | independent         | intracellular protein transport | SNA9                                 |
| Rpl37                                | 5E-02                       | 4E-05                  | dependent           | 60S ribosomal protein L37 | Rpi37                                 |
| **Control pro-survival genes**       |                             |                        |                     |                          |                                      |
| th(diap-1) a                         | 9E-04                       | 1E-05                  | independent         | anti-apoptosis           |                                      |
| E75                                  | 7E-04                       | 3E-01                  | independent         | DNA binding: steroid hormone receptor | NR1D1                                |
| **Pro-survival genes (ecdysone dependent and independent)** |                             |                        |                     |                          |                                      |
| sin3A                                 | 3E-04                       | 1E-02                  | independent         | transcription factor     | SIN3B                                 |
| S6K c                                | 6E-04                       | 3E-03                  | independent         | positive regulator of cell growth | RPS6KB1                              |
| Rpn2 a                               | 8E-04                       | 6E-04                  | independent         | endopeptidase            | PSMD1; RYR2                          |
| Pros26.4 a                           | 9E-04                       | 9E-04                  | independent         | endopeptidase            | PSMC1                                 |
| Ras85D a                             | 2E-03                       | 2E-01                  | dependent           | G-coupled signaling, anti-apoptotic | HRAS                                 |
| Smr a                                | 2E-03                       | 4E-03                  | independent         | DNA binding: protein binding | NCOR1                                 |
| Vps32 a                              | 3E-03                       | 6E-03                  | independent         | carrier activity         | CHMP4B                                |
| Tbp-1 a                              | 4E-03                       | 9E-04                  | independent         | endopeptidase            | PSMC3                                 |
| Tor z                                | 6E-03                       | 6E-03                  | independent         | protein kinase, cell growth, autophagy | FRAP1, mTOR                          |
| CG33087                              | 7E-03                       | 4E-01                  | dependent           | Ca++ ion binding: ATPase, LDL receptor activity | LRP1                                 |
| Indy                                 | 7E-03                       | 7E-03                  | independent         | tricarboxylic acid transporter | SLC13A2                              |
| Kap-n3 a                             | 7E-03                       | 3E-05                  | independent         | protein carrier          | KPNA3                                 |
| CG7466                               | 9E-03                       | 7E-03                  | independent         | receptor binding; cell-cell adhesion | MEGF8                                 |
| CEP                                  | 1E-02                       | 3E-01                  | dependent           | cathepsin L activity; proteolysis | CTSL                                  |
| CG32016                              | 2E-02                       | 8E-02                  | dependent           | unknown                  |                                      |
| HmgD a                               | 2E-02                       | 3E-03                  | independent         | DNA binding activity     |                                      |
| CG13784                              | 7E-02                       | 1E-01                  | dependent           | unknown                  |                                      |
| CG15239                              | 4E-02                       | 1E-03                  | independent         | unknown                  |                                      |

Gene symbols, CG numbers (column 1), and functions (column 5) are from Flybase [58]. The indicated Ortholog symbols (column 6) are from the HUGO Gene Nomenclature Committee (HGNC). P-values in columns 2 and 3 were calculated by comparing the WST-1 reading (A450–A650) from RNAi of the gene of interest to the WST-1 reading from RNAi of the human (Hs) negative control (NM_138278). Ecdysone dependent means that the observed viability effects of RNAi depended on the presence of ecdysone. Italicized and bolded P-values in column 3 indicate that these genes showed a pro-survival effect in the absence of ecdysone. Each RNAi treatment had three replicates and the assay was conducted at least twice. Superscript symbols a, b, and c indicate that these genes were identified in other related RNAi screens; a = Boutrous et al., 2004 [49]; b = Björklund et al., 2006 [47]; c = Bettencourt Dias et al., 2004 [57].

doi:10.1371/journal.pgen.1000379.t001

Based on genome-wide transcript and protein expression studies conducted previously in *Drosophila* larval salivary glands [23,24,40], there are a large number of genes and proteins that could affect ecdysone-mediated PCD but have not been tested functionally. Here, we conducted a systematic study of 460 of these genes which included all of the annotated genes from our previous study [24] that showed a significant (p≤0.05 and 5 fold difference) increase or decrease in expression levels in salivary glands immediately prior to PCD. The WST-1 assay was used as a primary screen to assess effects of the 460 dsRNAs on cell viability (Table S1). Using this assay, we identified five genes reported already to have a pro-survival role based on a previous RNAi screen [47,49,57]; (Table 1 and Table S1). In addition, we identified and validated another 20 genes with corresponding dsRNAs that significantly increased or decreased cell viability (Table 1). All of these 20 genes were validated with completely non-overlapping dsRNAs. In total, our final gene set for further analyses consisted of 18 genes with corresponding dsRNAs that...
resulted in reduced viability (hereafter referred to as pro-survival genes) and 7 genes with corresponding dsRNAs that resulted in increased viability (hereafter referred to as candidate pro-death genes).

**Identified Pro-Survival Genes Act in an Ecdysone-Dependent or Ecdysone-Independent Manner**

To determine which genes are regulated by the ecdysone signaling pathway, we investigated whether the decreased cell viability phenotype caused by RNAi knock-down of the 18 pro-survival gene products was ecdysone dependent. We treated the cells with dsRNA and assessed cell viability with and without ecdysone treatment. This analysis resulted in the identification of five ecdysone dependent pro-survival genes (CG33087, CG13784, CG32016, Ras85D, Cp1; Table 1). dsRNAs corresponding to these five genes reduced cell viability only in the presence of ecdysone and did not affect viability of l(2)mbn cells in the absence of ecdysone. Of the five genes identified, three (CG33087, CG13784, CG32016) were uncharacterized previously. Of these three genes, two (CG13784, CG32016) do not have any recognizable protein domain or predicted gene function (FlyBase) [58]. We confirmed the ecdysone dependent pro-survival effect of two (CG32016, Cp1) of the 5 identified genes in another *Drosophila* cell line, S2; the other three ecdysone dependent genes identified in l(2)mbn cells did not significantly affect S2 cell viability in the presence of ecdysone (Table S2). dsRNA corresponding to 13 other genes (Table 1) reduced viability of l(2)mbn cells following ecdysone treatment. However, a decreased viability phenotype, as assessed by WST-1, was also observed for these 13 dsRNAs in l(2)mbn cells in the absence of ecdysone. Nine of the 13 dsRNAs showed similar viability effects in S2 cells in the presence or absence of ecdysone (Table S2). We initially categorized the 13 genes as ecdysone independent pro-survival genes. Among this group of genes,

| Table 2. Ecdysone dependent and ecdysone independent death-related effects identified by the TUNEL assay. |
|---------------------------------------------------------------|
| **Gene Targeted** | **Ecdysone treatment** | **No ecdysone treatment** |
| | Percent dead cells * | TUNEL Assay | p-value | Percent dead cells * | TUNEL Assay | p-value |
| No Ecd or dsRNA treatment | | | | 11 |
| Ecdysone treatment | 54 |
| NM, 138278 | 56 | control | 9 | control |
| **Control Pro-death genes** | | | | |
| bR-C | 13 | – – | 4E-05 |
| dronc (Nc) | 24 | – | 3E-02 |
| **Control Pro-survival genes** | | | | |
| th (diap-1) | 76.8 | + + | 4E-06 |
| **Candidate Pro-death Genes** | | | | |
| Hid | 13 | – – | 3E-05 |
| RpLP1 | 34 | – – | 1E-04 |
| Rpl13A | 38 | – – | 3E-04 |
| EcR | 9 | – – | 5E-03 |
| Rpl37 | 29 | – – | 5E-03 |
| Rp55 | 44 | – – | 8E-03 |
| Sox14 | 37 | – | 3E-02 |
| SH3PX1 | 47 | – | 3E-02 |
| RpS6 | 54 | NC | 5E-01 |
| **Pro-survival Genes** | | | | |
| **Group A: Increased TUNEL positive cells only in the presence of ecdysone** | | | | |
| Kap-p3 | 85 | ++ | 4E-05 | 14 | NC | 1E-01 |
| CG32016 | 72 | ++ | 6E-03 | 21 | NC | 5E-02 |
| Ras85D | 65 | ++ | 1E-02 | 11 | NC | 4E-01 |
| Smr | 69 | + | 2E-02 | 17 | NC | 1E-01 |
| **Group B: Increased TUNEL positive cells in the absence or presence of ecdysone** | | | | |
| S6K | 72 | + | 2E-02 | 21 | + | 5E-02 |
| Pros26.4 | 65 | + | 4E-02 | 31 | ++ | 3E-04 |
| **Group C: Increased TUNEL positive cells only in the absence of ecdysone** | | | | |
| Tor | 62 | NC | 5E-02 | 23 | ++ | 2E-03 |
| sin3A | 64 | NC | 1E-01 | 20 | + | 3E-02 |

Gene names are from Flybase [58].

*Percent dead cells = Number of TUNEL positive cells/Number of DAPI positive cells ×100. P values were calculated by comparing the percent dead cells of each RNAi treatment to the human control (NM_138278) RNAi treatment (++ = significantly increased TUNEL with p<0.01; ++ = significantly increased TUNEL with p<0.05; + = significantly decreased TUNEL with p<0.01; – = significantly decreased TUNEL with p<0.01; NC = no significant change in TUNEL). doi:10.1371/journal.pgen.1000379.t002
dsRNA corresponding to Kap-α3 resulted in different phenotypes, as assessed initially by cell morphology (Figure 3), in the absence and presence of ecdysone. In the absence of ecdysone, Kap-α3 dsRNA did not result in detectable apoptotic bodies (up to 72 hrs), but in the presence of ecdysone and as early as 48 hrs following treatment, the same dsRNA resulted in a dramatic increase in apoptotic bodies compared to controls (Figure 3; Table 2). This result indicates that while the overall survival effect of this gene product may be ecdysone independent, its mechanism of action differs depending on the presence or absence of ecdysone.

Figure 2. Overview of RNAi screen in l(2)mbn cells. A set of 460 genes which included known transcription factors, cell death and autophagy genes, as well as genes associated transcriptionally with ecdysone-induced cell death of the Drosophila salivary gland were chosen for this functional study. In our initial screen, cells were treated with ecdysone and dsRNA corresponding to each of these genes. A viability assay (WST-1) was used to identify genes with potential pro-death or pro-survival functions, and microscopy was used to visualize cell morphology. For the reproducible positive hits, a second dsRNA was used to confirm the viability phenotype. The screen identified 7 potential pro-death and 18 pro-survival genes. The RNAi/WST-1 screen was repeated with and without ecdysone to determine ecdysone dependency for the 25 identified genes. TUNEL/DAPI and BrdU assays were performed to identify genes with cell death and/or cell proliferation related effects.

doi:10.1371/journal.pgen.1000379.g002
Figure 3. Cellular morphology of l(2)mbn cells after dsRNA treatment. Cellular phenotypes were visualized 3 days after dsRNA treatment in the presence or absence of ecdysone. The l(2)mbn cells with no ecdysone and no dsRNA (No treatment) were round and uniform in size and shape. l(2)mbn cells treated with ecdysone (not shown) or ecdysone-human dsRNA NM_138278-negative control changed in shape, from round to spindle forms with extensions (†). Large cells with phagocytosed material (△) and apoptotic bodies (□) were also observed. RNAi of EcR, dronc and Sox14 each increased viability of the ecdysone treated cells, but their resulting morphologies were distinct. The % of observed spindle shaped cells (top right corner) was quantitated for dsRNAs corresponding to the genes indicated. RNAi of EcR inhibited spindle shape formation (1+/−2% of the cells were spindle shaped), cells remained rounded, and no apoptotic bodies were found. RNAi of dronc inhibited apoptotic body formation, but cells became spindle shaped (20+/−8%). Also, signs of necrosis such as inflated and seemingly empty cells and cell fragments were observed (▼). RNAi of Sox14 showed few apoptotic bodies and 30+/−8% of the cells were spindle-shaped. RNAi of diap1 resulted in formation of numerous apoptotic bodies within 24 hr and no spindle shaped cells were found. RNAi of Rpn2 showed numerous apoptotic bodies in the presence and absence of ecdysone, while RNAi of Kap-a3 showed a dramatic increase in apoptotic bodies only in the presence of ecdysone (see Table 2 for quantitation of TUNEL positive cells). doi:10.1371/journal.pgen.1000379.g003

TUNEL and BrdU assays distinguish pro-survival genes associated with cell death inhibition

To determine whether the decreased viability of cells treated with ecdysone and dsRNA corresponding to the pro-survival genes is due at least in part to increased cell death, we performed the TUNEL/DAPI assay for representative genes from this category. We treated cells with dsRNA of two ecdysone dependent (CG32916 and Ras83D) and six ecdysone independent (Kap-a3, Pros26.4, S6K, and Tor) genes in the presence and absence of ecdysone and quantified the percent TUNEL positive cells. RNAi of six genes (CG32916, Ras83D, Kap-a3, Pros26.4, S6K, and Tor) increased significantly the percentage of TUNEL positive cells (p=0.05) in the presence of ecdysone (Groups A and B, Table 2, Figure 4), indicating a potential death inhibitory pro-survival role. RNAi of Sin3A and Tor did not significantly (p>0.05) increase the percentage of TUNEL positive cells in the presence of ecdysone, indicating that their pro-survival effects in this context are likely not due to an inhibition of cell death (Group C, Table 2).

However, RNAi of these same two genes did result in an increase in percent TUNEL positive cells in the absence of ecdysone compared to the controls (Table 2). In contrast, our TUNEL/DAPI assay indicated that knock-down of Kap-a3 and Ssr by RNAi increased TUNEL positive cells only in the presence of ecdysone (Group A, Table 2). This result is in agreement with the previously observed increase in apoptotic bodies found only in the presence of ecdysone (Figure 3). The reduced viability caused by RNAi of Kap-a3, and Ssr in the absence of ecdysone appears not to be death-related and may instead be due to inhibition of cell proliferation. To test this possibility, we conducted a BrdU incorporation assay which indicated reduced proliferation in Kap-a3-RNAi but not in Ssr-RNAi treated cells compared to control-RNAi treated cells (p<0.05; Figure S2). RNAi of Pros26.4 and S6K resulted in an increase in TUNEL positive cells (p=0.05) both in the absence and presence of ecdysone, distinguishing them as ecdysone independent and potential negative regulators of cell death. The TUNEL/DAPI assay also confirmed the ecdysone dependent and potential death inhibitory survival role of CG32916 and Ras83D (Group A, Table 2). Consistent with WST-1 findings, the BrdU incorporation assay for these two dsRNAs indicated no significant change in proliferation (p=0.5 and 0.4, respectively; Figure S2) in the absence of ecdysone. In summary, based on WST-1, TUNEL and BrdU assays, we conclude that CG32916, Ras83D, Kap-a3, and Ssr, are ecdysone dependent pro-survival genes that result in decreased cell death, and Pros26.4, S6K, Tor, and Sin3A are ecdysone independent pro-survival genes that result in decreased cell death. The observed effects on cell death following RNAi of these genes may be directly or indirectly related to gene function.

TUNEL Assay Validates Genes with a Pro-Death Function in Ecdysone-Mediated l(2)mbn Cell Death

Our RNAi study identified seven candidate pro-death genes, comprised of two 40S ribosomal genes (RpS5 and RpS6), three 60S ribosomal genes (RpL13A, RpL37 and RpL7P), one transcription factor Sox box protein (Sox14) and one sorting nexin-like gene (SH3P3X). To determine whether their potential pro-death effects are ecdysone dependent, we performed RNAi assays with and
without ecdysone. Consistent with observations by others [49], dsRNAs corresponding to the ribosomal genes had the opposite effect in the absence of ecdysone, resulting in a significant reduction in cell viability (Table 1, column 3, bold and italicized) when compared to control cells. In agreement with the cell viability assay, the BrdU assay showed reduced proliferation in the ribosomal gene-RNAi treated cells in the absence of ecdysone (p<0.05; Figure S2). To confirm the putative pro-death role of the ribosomal genes observed in the presence of ecdysone in l(2)mbn cells, we employed the TUNEL/DAPI assay as described above.

Figure 4. TUNEL assay identifies genes with cell death-related effects. DAPI staining (magenta) and TUNEL assays (green) were performed 3 days following addition of dsRNA and ecdysone to l(2)mbn cells. The cells with no ecdysone and no dsRNA (No treatment) showed a background level of 11±4% TUNEL positivity (Panel A). Ecdysone (not shown) and ecdysone plus the human dsRNA NM_138278-negative control resulted in an increase in TUNEL positive cells (56±5%; Panel B). dsRNA corresponding to diap1 (control gene) increased TUNEL positive cells without (not shown) or with ecdysone treatment (77±6%; Panel C). Ecdysone plus RNAi of Sox14 (Panel D) or EcR (Panel E) resulted in decreased TUNEL positive cells (37±6% and 9±0.5%, respectively), whereas ecdysone plus RNAi of a novel gene CG32016 (Panel F) increased TUNEL positive cells (72±8%) compared to the negative control.

doi:10.1371/journal.pgen.1000379.g004
Knock-down of all ribosomal genes tested, with the exception of RpS6, resulted in a decrease in the percent TUNEL positive cells (Table 2) following edcsyne treatment, indicating that RpS5, RpL13A, RpL37 and RpL41 have a pro-death related function in l(2)mbn edcsyne-mediated death. The TUNEL/DAPI assay also indicated that the transcription factor Sox14, and the sorting nexin-like gene SH3PX1 act as pro-death genes (Table 2). Therefore, our RNAi study which employed both cell viability (WST-1) and cell death TUNEL/DAPI assays identified six new genes (RpS5, RpL13A, RpL37 and RpL41, SH3PX1, Sox14) required for edcsyne-mediated cell death in l(2)mbn cells.

Transcription Factor Sox14 Is Induced by Edcsyne and Its Overexpression Is Sufficient to Induce Apoptosis In Vitro

To determine whether Sox14 expression is induced by edcsyne, we treated both l(2)mbn and S2 cells with edcsyne and determined the expression levels of Sox14 by QRT-PCR. As shown in Figure 5A, edcsyne treatment resulted in a 5 fold and 4 fold increase in expression of Sox14 in l(2)mbn and S2 cells, respectively.

To determine whether Sox14 is sufficient to decrease cell viability, we overexpressed C and N-terminal FLAG tagged Sox14 protein in l(2)mbn cells and measured cell viability using the WST-1 assay. Overexpression of Sox14 reduced cell viability, detectable 48 hrs following transfection (Figure 5B). By approximately 96 hrs after transfection, apoptotic bodies were evident in Sox14 overexpressing cell cultures but not in control cells transfected with empty vector (data not shown). These results indicate that Sox14 expression is sufficient to induce apoptosis in l(2)mbn cells.

Sox14-RNAi Animals Have Defects in Larval Midgut and Salivary Gland Destruction

To examine the function of Sox14 in vivo, we used a TabulinGAL4 driver (Tab-GAL4) to ubiquitously express Sox14 dsRNA (Tab-GAL4/Sox14-RNAi; referred to as Tab-Sox14-RNAi). QRT-PCR analysis using RNA from Tab-Sox14-RNAi wandering larvae and 0 hrs APF pupae showed a reduction in Sox14 transcripts of 89+/−2% and 91+/−2%, respectively, compared to wild-type control animals (Figure 5C). The Tab-Sox14-RNAi animals demonstrated lethality at 3rd instar larval, pupal or pharate adult stages. During pupation, the Tab-Sox14-RNAi animals appeared normal but the dorsal tracheal trunks showed severely distorted taenidial folds, collapse of the tracheal cuticle and blackening of the cuticle (Figure 6B). Tab-Sox14-RNAi animals did not cease, but we dissected out pharate animals and found obvious alterations in the notum (malformed; split) and bristles (missing and mis-oriented) (Figure 6C). The Sox14-related cellular alterations giving rise to these defects remain to be determined.

To initiate investigations of Sox14 in programmed cell death, we first examined the larval midgut (Figure 6D and E); this tissue was examined since most (86%) Tab-Sox14-RNAi pupae persist past the normal stage of larval midgut cell death. By 4 hrs APF, the proventriculus is significantly reduced in size and the gastric caeca are no longer detectable in wild-type animals. Head eversion occurs at approximately 10–12 hrs APF, at which time point the larval midgut is entirely destroyed, compressed and surrounded by the adult midgut [9]. As expected, in control animals (Tab-GAL4/+; designated wild-type or wt) we observed midgut condensation by 4 hrs APF and the gastric caeca were not detectable after 7 hrs APF (n = 10) (Figure 6E). The wild type larval midgut appeared degraded and the remnants were found within the adult midgut by 12 hrs APF (n = 5). Similar to BR-C mutants [10], the Tab-Sox14-RNAi pupae showed some condensation of midguts, but a remaining proventriculus and gastric caeca remnants were still observed even after 7–12 hrs APF (n = 12) (Figure 6E). A remaining proventriculus and gastric caeca remnants were observed even in animals that had clearly undergone head eversion (ie. 10–12 hrs APF). These observations indicate that reduced Sox14 expression results in partially defective larval midgut cell death and thus Sox14 is normally required for complete destruction of the larval midgut.

To examine the role of Sox14 in salivary gland cell death, we first examined salivary glands from head-everted Tab-Sox14-RNAi pupae (n = 23; equivalent to >30 hrs APF at 25°C based on incubation time). At this timepoint, all 25 animals still had intact salivary glands. However, since Tab-Sox14-RNAi animals arrest at various developmental ages following head eversion, we used retinal pattern formation [59] as an independent morphological marker to aid in the developmental staging. Retinas were dissected and stained with phalloidin to visualize ommatidial patterning [60]. All 23 animals had fully everted eye discs consistent with development to at least 12 hrs APF (at 25°C), and 8 animals had retinas with ommatidial patterning indicative of development to at least 22 hrs APF at 25°C [59]. Of these 8 animals, ommatidial patterning indicated that 5 developed to at least 30 hrs APF at 25°C (e.g. Figure 7A). In rare instances (n = 6 out of more than 100 pharate adults dissected), we were able to dissect intact salivary glands from Tab-Sox14-RNAi pharate adults with darkened wings and red eyes indicative of development to approximately 100 hrs APF (25°C) [59]. To further analyze Sox14 function in salivary gland cell death, we employed a salivary gland GAL4 driver (D59-GAL4) to express Sox14 dsRNA. A single copy of the driver did not result in a phenotype, but two copies of the driver (D59-GAL4/D59-GAL4; Sox14-RNAi/TM6B) resulted in a delay in salivary gland cell death compared to control animals (D59-GAL4/D59-GAL4; MKRS/TM6B) (Figure 7B–D). In the control animals, TUNEL positive nuclei were prevalent in salivary glands equivalent to 16–17 hrs APF at 25°C (ie. 30–32 hrs APF at 18°C) but were not observed in salivary glands from D59-Sox14-RNAi or Tab-Sox14-RNAi animals at a comparable or later stage, respectively (Figure 7A–C). Together, these results indicate that reduced levels of Sox14 expression result in either a delay or inhibition of salivary gland cell death, and thus Sox14 functions as a positive regulator of salivary gland cell death.

To help place Sox14 within the context of the known signaling pathways required for edcsyne-mediated cell death, we examined gene expression in Tab-Sox14-RNAi animals. Transcript levels of two apoptosis effectors, npr and dronc, and two edcsyne regulated transcription factors, E93 and BR-C, were examined in Tab-Sox14-RNAi wandering larvae and 0 hr APF pupae and compared to controls. While BR-C showed no changes in expression levels between control and Tab-Sox14-RNAi animals at both stages examined, npr, dronc and E93 transcripts were reduced in Tab-Sox14-RNAi 0 hr APF pupae compared to controls (Figure 7E). These results support a pro-death role for Sox14, and indicate that Sox14 acts upstream of npr, dronc and E93 and either downstream or in parallel to BR-C. Future studies are required to determine whether Sox14 directly regulates the transcription of any of these genes, and whether the hierarchical position of Sox14 is conserved in various developmental stages and tissues.
Figure 5. Expression analyses of Sox14 in Drosophila cell lines and Sox14-RNAi animals. (A) QRT-PCR expression profiling of l(2)mbn and S2 cells treated with 10 μM ecdysone for 72 hrs showed a 5 fold and 4 fold increase, respectively, in Sox14 transcripts compared to untreated control cells. Expression of Sox14 was normalized to the housekeeping gene rp49. (B) l(2)mbn cells transfected with either N- or C-terminal FLAG tagged Sox14 constructs showed decreased cell viability after 48 hrs compared to cells transfected with the control vector only (p<0.001). (C) Sox14 expression in wild-type and Sox14-RNAi whole animals. In control wild type (wt; strain w1118) whole animals, increased transcript levels of Sox14 were observed at 0 hrs APF compared to wandering larvae. In Tub-Sox14-RNAi (Tubulin-GAL4/Sox14-RNAi) animals, Sox14 transcripts were dramatically reduced at both stages compared to the control (89+/−2% and 91+/−2% reduction in transcript levels at the wandering larva and 0 hrs APF stages, respectively). Error bars in (A)–(C) represent the SD of triplicate samples.

doi:10.1371/journal.pgen.1000379.g005

Discussion

We performed an RNAi screen as a means of gaining new molecular insights into ecdysone induced cell death and cell survival signaling pathways. We enriched for the identification of ecdysone-dependent genes by targeting genes that were differentially expressed in Drosophila larval salivary glands immediately prior to ecdysone-induced cell death [23,24]. In total, we verified functionally the pro-death effects of six genes and the pro-survival effects of 16 genes, and further characterized their functions on the basis of ecdysone dependency and cell death effects. More detailed examination of one gene, Sox14, showed that it was induced by ecdyson and its expression was sufficient to induce apoptosis in vitro. Studies in vivo revealed a role for Sox14 in larval midgut and salivary gland cell death.

Potential off-target effects can be a significant issue in any RNAi screen especially when long dsRNAs are used [61,62]. Although Drosophila does not have interferon responses as observed in mammals, short dsRNAs (≥19 nt) produced by Dicer processing that are perfect matches to non-target specific transcripts are the likely source of off-target effects [61–64]. To help eliminate potential false positives due to off-target effects or experimental noise, we designed a second dsRNA, free of predicted off-target effects and completely non-overlapping with the first dsRNA [65] (in all but one case – see Materials and Methods). For a gene to be considered further, both of its dsRNAs had to produce an effect in the same direction with a p-value of ≤0.05. While we may have eliminated some false negatives due to insufficient RNAi knockdown by this screening strategy, these stringent criteria enabled us to produce a highly reliable final list of candidate genes for further study. Many, but not all, of the dsRNAs corresponding to these candidate genes had similar effects on viability in l(2)mbn and S2 cell lines (Table S2). The observed differences may be attributable to the different genotypes of these cell lines. Since both l(2)mbn and S2 cell lines are polyclonal, we also cannot rule out the possibility of an inhomogenous response to the dsRNAs tested. This could affect the overall detectable response to RNAi treatment and thus is another possible reason why results could differ in these or alternate cell lines.

Recently, a role for Drosophila autophagy genes atg1, atg2, atg3, atg6, atg7, atg8a, and atg12 in salivary gland degradation has been demonstrated [66]. Our study did not find a death related role for autophagy genes in l(2)mbn cells in the presence of ecdysone. It is possible that these genes do not have an essential death or survival related role under the conditions we tested. Since our screen was optimized to detect effects of genes that are dependent on ecdysone-regulated transcription, we cannot rule out the possibility that additional genes impacting ecdysone-mediated PCD may be detected under different experimental conditions. However, in the absence of ecdysone, knock down of several Atg genes (atg2, atg3, atg5, atg6, atg7, atg8a, atg8b) resulted in decreased cell viabiliy (Figure S1) indicating a potential pro-survival role for these genes.

Our screen was validated by identification of known genes and biochemical complexes with previously established cell survival or cell death phenotypes. For example, Ras35D promotes cell survival in Drosophila by down-regulating hid expression and activity [67,68] in vivo. Consistent with these findings, we discovered that decreased Ras35D transcripts resulted in reduced cell survival in an ecdysone dependent manner, while knockdown of hid resulted in a phenotype of increased cell survival. These results suggest that Ras pathway mediated inhibition of Hid activity may exist in the ecdysone signaling pathway. We also identified Smr, a co-repressor, and dSin3A, a transcriptional regulator, that associate with each other to mediate the transcriptional silencing of the EcR:USP complex. Addition of ecdysone completely dissociates Smr from the EcR:USP heterodimer complex and activates EcR:USP mediated transcription. Elimination of repression by Smr/dSin3A on EcR:USP activity resulted in lethality in vivo [69]. Based on these observations, we predicted that reduced expression of either Smr or dSin3A or both by RNAi in our system would release, as with ecdysone, the repression caused by these gene products on the EcR:USP complex, resulting in increased EcR:USP activation and subsequent increased cell death. As we expected, our cell viability/TUNEL assays in l(2)mbn cells indicated clearly that knock-down of Smr transcripts resulted in increased cell death in an ecdysone dependent manner (Table 2). The identification of such known ecdysone signaling complexes demonstrates that our assay is a viable method for functional verification and initial characterization of genes involved in ecdysone-mediated death/survival pathways.

The predicted or known function of several pro-survival genes identified in our screen (Pros26.4, Rpn2, Thp-1 and Gpl) was associated with protein degradation processes. Under stress conditions, down regulation of gene products associated with protein degradation processes could impair energy production and, therefore, reduce the survival of the cell/organism. The 26S proteasome complex, a major site of protein degradation, is made up of two multi-subunit sub complexes, namely the 20S Proteasome and PA700 (19S complex). The identified pro-survival genes, Pros26.4, Rpn2, and Thp-1 all belong to the PA700 subunit of the 26S proteasome complex. Proteasome function is required for cell proliferation [70] and silencing the expression of gene products belonging to the PA700 complex by RNAi reduced cell proliferation and induced apoptosis in S2 cells [49,71]. Consistent with these previous findings, our results indicated that Pros26.4, Rpn2, and Thp-1 knockdown led to reduced viability of l(2)mbn and S2 cells both in the presence and absence of ecdysone. In our RNAi screen, the pro-survival genes that were associated previously with protein degradation (as above) or protein transport (Kap-93) were significantly up-regulated prior to larval salivary gland histolysis [24]. During PCD, anabolic processes are reduced and, therefore, a replenishable source of carbohydrates is unavailable for energy production. Thus, it is possible that ecdysone may activate protein degradation processes in salivary glands to produce energy to complete the death process.

Our RNAi screen identified five previously uncharacterized genes (CG13784, CG13239, CG32016, CG33087, and CG7466) as pro-survival genes. Among these, CG13784, CG32016 and CG33087 were ecdysone dependent for their pro-survival role. Further studies are required to determine whether these three
genes affect survival in response to other agents that induce cell death; our preliminary data (not shown) indicates that they do not have any effects on staurosporine induced cell death. The products of CG33087 (calcium ion binding; ATPase activity; low-density lipoprotein receptor activity) and CG7466 (receptor binding; cell-cell adhesion) have predicted functions based on protein domains but CG13784, CG15239, and CG32016 have no illuminating sequence characteristics. We further characterized CG32016 in l(2)mbn cells by the TUNEL assay as in both the presence and absence of ecdysone. Knock-down of CG32016 resulted in increased TUNEL positive cells only in the presence of ecdysone, indicating a potential cell death-related, ecdysone-dependent pro-survival role. We are the first to associate a function with these previously uncharacterized gene products (CG13784, CG15239, CG32016, CG33087, and CG7466); additional studies will be required to elucidate their specific positions and functions in response to ecdysone.

Of the 25 genes that were identified in our screen, seven genes (Table 1) were identified as potential pro-death genes. Of these seven genes, five were ribosomal genes. In Drosophila, 38 small (40S) and 49 large (60S) ribosomal proteins have been identified [72]; the small ribosomal subunits belong to the eukaryotic pre-initiation complex and the large ribosomal subunits are usually involved in translation. We tested in our RNAi screen the five ribosomal genes that were differentially expressed in the Drosophila larval salivary glands immediately prior to PCD [24]. RNAi of both small ribosomal genes (RpS5, RpS6) and large ribosomal genes (Rpl15A, Rpl37 and Rplp1) resulted in increased cell viability of ecdysone treated l(2)mbn cells, indicating that these genes may have a pro-death role in the presence of ecdysone. Further, with staurosporine treatment (data not shown), RNAi of these ribosomal genes resulted in reduced cell viability, indicating that ecdysone is indeed required for the increased viability effect of these dsRNAs l(2)mbn cells. Ecdysone treatment induces transcription of pro-death genes such as Br-C, dcr-2, epr and hid, and ribosomal gene products are required for their translation. Thus, knocking down ribosomal gene products by RNAi may affect efficient translation of pro-death genes leading to the observed phenotype of increased viability. However, in S2 cells, knock-down of these ribosomal genes in the presence of ecdysone did not increase cell viability but rather significantly decreased viability (Table S2); further studies are required to understand these cell line dependent effects. In the absence of ecdysone, RNAi of these same ribosomal genes resulted in reduced viability in both l(2)mbn and S2 cells, supporting a pro-survival role under these conditions. This pro-survival effect is similar to that reported in S2 and Kc cells by others [47,49]. A pro-survival function of ribosomal proteins in the absence of ecdysone is in agreement with the key role they play in protein-synthesis and, therefore, in cell growth and cell proliferation.

Our screen identified two additional gene products required for ecdysone-mediated cell death: i) dSH3PX1, involved in intracellular protein transport and resembling a sorting nexin with an NH2-terminal SH3 domain and a central phox homology (PX) domain [73,74], and ii) Sox box protein 14 (Sox14), a High mobility group (HMG) box-containing transcription factor related to the mammalian sex determining factor, SRY [75]. dSH3PX1 acts as a binding partner for the non-receptor Cdc-42 associated kinase (ACK) in Drosophila [76]. A similar interaction between ACK2 and SH3PX1 (also called SNX9) occurs also in mammals where further studies showed that phosphorylation of SH3PX1 by ACK2 regulates the degradation of EGF receptor [77]. Thus, it is possible that the knockdown of dSH3PX1 by RNAi in l(2)mbn cells results in decreased cell death through enhanced EGF receptor-
mediated cell survival signaling. Alternatively, the role of dSH3PX1 in cell death may be related to its associations with proteins involved in receptor trafficking and/or cytoskeletal rearrangements [78].

Our in vitro and in vivo analyses also identified for the first time a pro-death role for the transcription factor Sox14. Previously [24] we determined that of 19 genes tested, just two genes, Sox14 and ask, were independent of E93 regulation in dying larval salivary glands. This previous finding indicates that Sox14 may act in parallel to E93 or may be acting upstream of E93 in the ecdysone induced cell death pathway. Our gene expression analyses reported here (Figure 7E) position Sox14 upstream of E93, and also upstream of rpr and dronc that are known to be regulated by E93 [27]. A recent microarray study conducted during Drosophila pupariation further supports this view as Sox14 was identified as an ecdysone primary-response regulatory gene [79]. Based on comparison of the HMG box region, Drosophila Sox14 is most similar to mouse Sox4 and human Sox1, 11 and 22 [75,80,81].

Sox proteins regulate multiple downstream targets and are involved in numerous developmental processes. In particular, human Sox 4 has been implicated in both the positive [82,83] and negative [84] regulation of apoptosis.

Our in vivo studies using a Sox14-RNAi construct support a pro-death role for Sox14 during Drosophila ecdysone-triggered larval midgut (Figure 6) and salivary gland cell death (Figure 7). During metamorphosis, the larval midgut disintegrates and a new adult gut is formed. These two events overlap and the adult gut encompasses disintegrating larval gut tissue [9]. In Tub-Sox14-RNAi (Tubulin-GAL4 / Sox14-RNAi) animals, adult midgut cells are visible at 4 hrs APF similar to wild type gut, but complete condensation of the proventriculus and gastric caeca were inhibited at least up to 12 hrs APF. This observation is similar to what was observed in BR-C mutants, but different from E93 mutants which showed defects in larval midgut compaction but not destruction of the proventriculus and gastric caeca [10]. Thus, the midgut cell death defective phenotype of Sox14 is again in agreement with our prediction that...
Sox14 is acting upstream of E93 and downstream or parallel to BR-C. Our results using both the Tub-Sox14-RNAi (tubulin GAL4 driver) and D59-Sox14-RNAi (salivary gland GAL4 driver) animals support a role for Sox14 as a positive regulator of salivary gland cell death. Cell death was delayed in D59-Sox14 RNAi animals and was either delayed or inhibited as late as the pharate adult stage in Tub-Sox14-RNAi animals. It is possible that the less severe phenotype in the D59-Sox14-RNAi animals is due to less efficient RNAi-mediated knockdown of Sox14, a notion that is supported by our observed dose-dependent effects of the D59-GAL4 driver. Given the predicted function of Sox14 as a transcription factor, it is particularly likely that even reduced amounts could still lead to some wild-type function. It is also possible that Sox14 functions in a partially redundant manner in both the midgut and salivary gland so that even a complete loss of function may lead to only a partial loss or delay in cell death. Null mutants of Sox14 would be valuable for future testing of these possibilities.

In addition to defects in midgut and salivary gland cell death, we observed tracheal defects in Tub-Sox14 RNAi animals, similar to defects observed in mutants of DHR3 which encodes an ecdysone responsive orphan nuclear receptor [85]. Preliminary examination of Tub-Sox14-RNAi pharate adults indicated additional roles for Sox14 in notum and bristle development. Future studies are required to determine the function of Sox14 in these and other tissues. Given its predicted role as a transcriptional regulator and its position in the ecdysone signaling cascade, it is likely that Sox14 will function in various cellular processes.

In summary, we developed an RNAi-based screening system to identify genes that are required for ecdysone-mediated cell death and survival pathways. Our screen identified known and novel components of the ecdysone signaling network that act as pro-death or pro-survival genes. In particular, we have shown that in some cases the function of a gene is dependent on ecdysone, or its mechanism of action is variable depending on the presence or absence of ecdysone. In vivo studies of Sox14 support a role in ecdysone-mediated cell death. Further characterization of the novel genes identified is necessary to elucidate their specific roles and positions in the ecdysone signaling network.

Materials and Methods

dsRNA Design and Synthesis

For the initial screen, individual PCR products up to 735 bp in length and containing coding sequences for the transcripts to be knocked-down (Table S1) were generated by RT-PCR using 500 ng of total RNA and Superscript one-step RT-PCR kit with platinum taq (Invitrogen). Each primer used in the RT-PCR contained a 5′ T7 RNA polymerase binding site (TAATAC-GACTCAGATATTG) followed by sequences specific for the targeted genes (see Table S1). RT-PCR products were isopropylmagnesium-precipitated and the entire product from each reaction was used as template for in vitro transcription reactions. In vitro transcription reactions were carried out using either Megascript T7 transcription kit (Ambion) or T7 RiboMax Express RNAi systems (Promega) according to the manufacturer’s instruction. dsRNAs synthetized were incubated at 65°C for 30 min followed by slow cooling to room temperature. dsRNAs were ethanol precipitated and resuspended in 50 μl nuclease free water. A 5 μl aliquot of 1/100 dilution was analysed by 1% agarose gel electrophoresis to determine the quality of 1% dsRNA. The dsRNAs were quantitated using a picoquant assay (Invitrogen) and concentrations adjusted to 100 ng/μl with nuclease free water.

For genes of interest identified in our initial screen (for complete list see Table S1), we designed a second non-overlapping dsRNA to confirm the observed phenotype. The German Cancer Research Center (DKFZ) EnoRNAi search tool (Off-Target Search Tool: http://www.dfkz.de/signaling2/e-rna/) [86] was used to search the RNAi probes for potential off-target effects using a 19 bp fragment length cut-off. Our final criteria for confirmation of RNAi effects was that for each gene, at least one of its dsRNAs had no predicted off-target effects (i.e., 100% specificity) and a second dsRNA had at least 90% specificity. Of the 20 genes that we confirmed by this method, 19 were represented by two dsRNAs that were completely non-overlapping. One additional gene confirmed by this method, RpL13A (CG1475), was represented by two dsRNAs that overlapped by 21 bp. Analysis of this 21 bp by the Off-Target Search Tool indicated 0 potential secondary targets.

Cell Culture and Ecdysone Treatment

l(2)mbn cells [42] and S2 cells (Invitrogen) were grown in Schneider’s (Invitrogen) medium supplemented with 10% FBS, 50 units/ml penicillin and 50 μg/ml streptomycin (Gibco-BRL) (hereafter referred to as Schneider’s medium+10%FBS) in 25-cm² suspension flasks (Sarstedt) at 25°C. All experiments were carried out 3 days after passage and the cells were discarded after 25 passages. 20-Hydroxyecdysone (ecdysone) was obtained from Sigma-Aldrich and resuspended in 95% ethanol at a concentration of 10 mM.

Quantitative RT-PCR

Three days after passage, cells were adjusted to 1×10⁶ cells/ml in ESF921 serum free media (Expression systems) and 3×10⁵ cells (333 μl) were seeded into each well of a 24 well plate. After one hour incubation, 667 μl of Schneider’s medium +10%FBS and ecdysone (10 μM final) (Sigma) was added to yield 1 ml culture in each well. Treated cells were incubated at 25°C for 24, 48, or 72 hours and 1 ml cultures were transferred to RNase free eppendorf tubes (Ambion) and cells were pelleted at 1000 rpm for 10 min. Cell pellets were lysed in 1 ml Trizol (Invitrogen) and total RNA was extracted according to manufacturer’s instructions. Isolated RNA was treated with RNase free DNase and 50 ng of total RNA was used in 15 μl QRT-PCR reactions. QRT-PCR reactions were carried out using the one-step SYBR green RT-PCR Reagent kit (Applied Biosystems) on an Applied Biosystems 7900 Sequence Detection System. Expression levels were calculated using the Comparative Cycle Threshold (CT) Method with a ribosomal housekeeping gene, as the reference for normalization. To determine the fold change in expression levels of known ecdysone signaling genes, cell death related genes and Sox14, following ecdysone treatment of l(2)mbn cells, the CT values were normalized to sp49 in the same sample (hereafter referred to as normalized CT values) for each gene, and were compared to the normalized CT values for the same gene from untreated control cells. Similarly, for the RNAi experiments, normalized CT values for each gene from ecdysone plus dsRNA-treated cells were compared to the normalized CT values from cells treated with ecdysone plus control human dsRNA, and the knock-down efficiency was calculated.

Knock-down efficiency = 100−[(Fold expression of targeted gene in dsRNA+ecdysone treated cells/Fold expression of targeted gene in ecdysone treated cells) ×100].

RNA Interference (RNAi) and Cell Viability Assays

For RNAi in l(2)mbn cells, a 33 μl volume of ESF921 media containing 3×10⁵ cells was seeded into each well of a 96 well plate...
for RNAi screens. Into each well, 500 ng–1000 ng of dsRNA in a 5 μl volume was added, and incubated for one hour at room temperature. The untreated control cells received 5 μl of nuclease free water. After one hour incubation, the cells received Schneider’s medium+10% FBS containing ecdysone (10 μM final) to yield a final 100 μl volume. Cells were incubated for 72 hours at 25°C and 10 μl of WST-1 reagent (Roche Scientific) was added. A450–A650 readings were taken after overnight incubation using a 96 well spectrophotometer VersaMax (Molecular Devices). A450–A650 readings of experimental samples were always compared to A450–A650 readings from cells treated with human dsRNA to control for any non-specific RNAi effects. RNAi experiments in S2 cells were essentially performed as for D. melanogaster cells with the following modifications: 1000–1500 ng of dsRNA in a 5–7.5 μl volume was added. Cells received 1 μM edysone overnight and then the edysone concentrations were increased to 10 μM final. Cells were incubated for a total of 48 hours (following addition of dsRNA) at 25°C and then 10 μl of WST-1 reagent was added. Assay readings were taken after overnight incubation. All samples were analyzed at least in triplicate.

Cell Morphology

To quantitate changes in cell shape, indicative of cell differentiation, following edysone treatment and RNAi, five images from two biological replicates were captured using an Axiovert 200 fluorescent microscope (Carl Zeiss). The observed number of spindle-shaped and rounded cells were counted manually. Percent spindle-shaped (differentiated) cells was calculated as the Number of spindle-shaped cells/Number of spindle-shaped cells+ Number of rounded cells*100.

Brdu (5-Bromo-2′-Deoxyuridine) Cell Proliferation Assay

RNAi experiments were carried out as described above and cell proliferation was determined using the Cell Proliferation BrdU ELISA kit (Roche Scientific). Cells received BrdU 64 hrs after dsRNA treatment and the cells were incubated for another 24 hrs. Cells were then processed as per manufacturer’s instructions and A370-A492 readings were taken using a 96 well spectrophotometer VersaMax (Molecular Devices). A370-A492 readings from experimental RNAi treatments were compared to the reading from cells treated with human dsRNA.

TUNEL Assay

For the TUNEL assay in vitro, RNAi experiments were carried out as described above except the cells were seeded into each well of 16-well CC2 coated chamber slides (Nunc). After 72 hours of respective treatment, cells received 100 μl of hypotonic solution (75 mM KCl) for 3–5 min at 25°C. Cells were then fixed with 3:1 methanol/acetic acid solution and air dried. Cells were washed with 1XPhosphate buffer saline (Sigma) and processed with TUNEL using the DeadEnd fluorometric tunel system (Promega). Cells were then fixed with Slowfade antifade with DAPI (Invitrogen) and viewed as described above. For transfection, 1 μg of plasmid DNA and 10 μl of Cellfectin (Invitrogen) were combined in 200 μl of serum-free Grace medium (Invitrogen) for 30 min. Immediately prior to transfection, 3×10^6 cells in 800 μl of Grace medium were prepared and incubated with the transfection medium (a total of 1 ml culture) overnight in a 24 well suspension culture plate (Sarstedt). Cells were equally split into two wells (500 μl each), and each well received 1 ml of Schneider+10%FBS medium. Cells were incubated for up to 96 hrs. At 48 and 72 hrs after transfection, 100 μl of cells were plated into a 96 well plate, 10 μl of WST-1 reagent was added, and absorbance readings were taken after overnight incubation. WST-1 readings of cells transfected with Sox14 expression constructs were compared to the cells transfected with empty vector (negative control). Transfected cells were monitored for the presence of apoptotic bodies up to 96 hrs after transfection. Samples were analyzed in triplicate, with two biological replicates of each construct.

Sox14-RNAi Lines

A UAS-Sox14-RNAi Drosophila line was obtained from the Vienna Drosophila RNAi Center. Heterozygous animals containing the Sox14-RNAi construct, balanced over TM6B, Tb^6 were crossed to a stock carrying the Tubulin-GAL4 driver (u+; Tub-GAL4/TM6B, Tb^6), derived from Tub-P-GAL4^DE3 (Bloomington stock centre) to drive the expression of Sox14-RNAi in vivo. The phenotype of the resulting F1 non-tubby progeny (Sox14-RNAi/Tub-GAL4; designated Tab-Sox14-RNAi) was compared to control animals (Tab-GAL4+/+) designated as wild-type (wt). The knock-down efficiency of Sox14 was determined by comparing transcript levels in Sox14-RNAi/Tub-GAL4 animals to the wt+/Tub-GAL4 animals using QRT-PCR as described above. For salivary gland-specific Sox14-
*RNAi* studies, animals containing *UAS-Sox14-RNAi* were crossed to a strain containing the D59 salivary gland driver [87] and a strain containing two copies of salivary gland driver and one copy of *UAS-Sox14-RNAi* was established (D59-GAL4/D59-GAL4, Sox14-RNAi/TM6B; designated D59-Sox14-RNAi). Control animals were D59-GAL4/D59-GAL4; MKRS/TM6B.

**Phalloidin Staining of Retinae**

Retinae were dissected from *Tab-Sox14-RNAi* animals following salivary gland dissection. Retinae were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 1% TritonX-100. Phalloidin-Rhodamine (Invitrogen) was used to stain and reveal dehye for 20 minutes and permeabilized with 1% TritonX-100. Retinae were mounted in Slowfade antifade with DAPI and viewed on a Zeiss Axioplan 2 microscope. Ommatidial organization, cell number and apical profile were used to assess developmental age [59].

**Statistical Analyses**

Probability p-values were calculated with Student’s t-test using two-tailed distribution and two-sample equal variance.

**Online Supplemental Material**

Table S1 provides a complete list of genes targeted by RNAi along with their primer sequences, amplicon lengths, cell viability results and p values. Table S2 includes a comparison of WST-1 assay results in l(2)mbn and S2 cells for the identified candidate pro-death and pro-survival genes. Figure S1 shows the cell viability effects of Atg gene RNAi in the absence of ecdysone and Figure S2 shows results of the BrdU cell proliferation assay.

**References**

1. Kies W, Gallaher B (1998) Hormonal control of programmed cell death/ apoptosis. Eur J Endocrinol 138: 497–501.
2. Winoto A, Littman DR (2002) Nuclear hormone receptors in T lymphocytes. Cell 109 Suppl: 85–87.
3. Smith SW, McLaughlin KA, Osborne BA (1995) Molecular events in thymocyte apoptosis. Curr Top Microbiol Immunol 200: 147–162.
4. Lockshin RA, Williams CMb (1965) Programmed Cell Death—IV. Cytology of Degeneration in the Intersegmental Muscles of the Pernyi Silkworm. J Insect Physiol 11: 123–133.
5. Lockshin RA, Williams CM (1965) Programmed cell death—IV. The influence of drugs on the breakdown of the intersegmental muscles of silkworms. J Insect Physiol 11: 1003–1009.
6. Lockshin RA, Williams CM (1965) Programmed cell death. V. Cytoytic enzymes in relation to the breakdown of the intersegmental muscles of silkworms. J Insect Physiol 11: 1251–1253.
7. Trumans JW, Talbot WS, Fahrbach SE, Hogness DS (1994) Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during Drosophila and Manduca development. Development 120: 219–234.
8. Jiang C, Barccheke EH, Thummel CS (1997) Steroid regulated programmed cell death during Drosophila metamorphosis. Development 124: 4673–4683.
9. Lee CY, Cooksey BA, Barccheke EH (2002) Steroid regulation of midgut cell death during Drosophila development. Dev Biol 250: 101–111.
10. Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. Science 267: 1456–1462.
11. Lee CY, Clough EA, Yellon P, Teslovich TM, Stepharn DA, et al. (2003) Genome-wide analyses of steroid- and radiation-triggered programmed cell death during Drosophila development. Dev Biol 253: 130–157.
12. Gorkis SM, Chitraranjan S, Pleasance ED, Freeman JD, Anderson CL, et al. (2003) A SAGE approach to discovery of genes involved in autophagic cell death. Curr Biol 13: 538–543.
13. Breaud J, Mcbrrie JR, Eadrizzi B, Thummel CS, Woodard CT (1999) The Drosophila beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol Cell 3: 143–149.
14. Heinrich VC, Rybczynski R, Gilbert LI (1999) Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. Vitarn Horm 55: 73–125.
15. Richards G (1997) The ecdysone regulatory cascades in Drosophila. Adv Dev Biol 5: 81–135.
16. Barccheke EH, Thummel CS (1995) The Drosophila E93 gene from the 93F early puff displays stage- and tissue-specific regulation by 20-hydroxyecdysone. Dev Biol 171: 85–97.
17. Jiang C, Lamblin AF, Steller H, Thummel CS (2000) A steroid-triggered transcriptional hierarchy controls salivary gland cell death during Drosophila metamorphosis. Mol Cell 5: 445–455.
18. Lee CY, Barccheke EH (2001) Steroid regulation of autophagic programmed cell death during development. Development 128: 1443–1455.
19. Lee CY, Clough EA, Yellon P, Teslovich TM, Stepharn DA, et al. (2003) Genome-wide analyses of steroid- and radiation-triggered programmed cell death during Drosophila development. Dev Biol 253: 130–157.
20. Breaud J, Mcbrrie JR, Eadrizzi B, Thummel CS, Woodard CT (1999) The Drosophila beta FTZ-F1 orphan nuclear receptor provides competence for stage-specific responses to the steroid hormone ecdysone. Mol Cell 3: 143–149.
21. Estudo La WK (1992) Mutations in a steroid hormone-regulated gene disrupt the metamorphosis of internal tissues in Drosophila: salivary glands, muscle, and gut. Wilhelm Roux’s Arch Dev Biol 201: 221–234.
22. Lee CY, Wendel DP, Reid P, Lam G, Thummel CS, et al. (2000) E93 directs steroid-triggered programmed cell death in Drosophila. Mol Cell 6: 433–443.
23. Thummel CS (2001) Steroid-triggered death by autophagy. Bioessays 23: 677–682.
24. Fischer JC, Thummel CS (1995) The ecdysone-inducible broad-complex and E74 early genes interact to regulate target gene transcription and Drosophila metamorphosis. Genetics 141: 1025–1035.
25. Fischer JC, Thummel CS (1995) The Drosophila E74 gene is required for the proper stage- and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. Development 121: 1411–1421.
26. Yin VP, Thummel CS, Bashirullah A (2007) Down-regulation of inhibitor of apoptosis levels provides competence for steroid-triggered cell death. J Cell Biol 176: 843–852.
27. Cao C, Liu Y, Lehmann M (2007) Fork head controls the timing and tissue selectivity of steroid-induced developmental cell death. J Cell Biol 176: 843–852.

**Supporting Information**

**Table S1** List of names, primer sequences, and viability results for genes targeted by RNAi.

Found at: doi:10.1371/journal.pgen.1000379.s001 (0.20 MB XLS)

**Table S2** Comparison of RNAi effects on cell viability and ecdysone dependency in l(2)mbn and S2 cells.

Found at: doi:10.1371/journal.pgen.1000379.s002 (0.07 MB DOC)

**Figure S1** RNAi of some Atg genes shows reduced viability in the absence of ecdysone.

Found at: doi:10.1371/journal.pgen.1000379.s003 (0.55 MB TIF)

**Figure S2** Analysis of cell proliferation by the BrdU incorporation assay in dsRNA-treated l(2)mbn cells.

Found at: doi:10.1371/journal.pgen.1000379.s004 (1.88 MB TIF)

**Acknowledgments**

We are grateful to A. Dorn for l(2)mbn cells, and to A. Dorn and S. Kumar for helpful advice on l(2)mbn cell culture. We thank D. Clark for the Tubulin-GAL4 driver. We thank M. Marra for helpful suggestions and G. Morin for critical reading of the manuscript.

**Author Contributions**

Conceived and designed the experiments: SC. Performed the experiments: SC MM YCCH JDF LD. Analyzed the data: SC MM YCCH JDF LD. Wrote the paper: SC. Assisted in the experimental design: SC. Edited the paper: SMG.
59. Cagan RL, Ready DF (1989) The emergence of order in the Drosophila pupal retina. Dev Biol 135: 1457-1465.

58. Crosky MA, Goodman JL, Sterlets VB, Zhang P, Gelbart WM (2007) FlyBase: A comprehensive database of the Drosophila melanogaster melanogaster genome. Nucleic Acids Res 35: D480-D491.

57. Dykes, M. S., Ruiz, J., Ewen, J. D., Bettenhauser, M., Nguyen, T., et al. (2010) Identification of Apd1 homologous ARG is required for motility, but not cell cycle progression. J Biol Chem 285: 9270-9277.

56. Eggert US, Kiger AA, Richter C, Perlman ZE, Perrimon N, et al. (2004) Parallel analysis of steroid-triggered autophagic programmed cell death during Drosophila development. Cell Death Differ 11: 916-923.

55. Strober W (1997) Trypan Blue Exclusion Test of Cell Viability. In: Coligan JE, Shehu S, Beck R, McMichael AJ, editors. Current Protocols in Immunology. John Wiley & Sons, Inc.

54. Foley E, O'Farrell PH (2004) Functional characterization. Cancer Res 66: 3434-3442.

53. Friesen SD, Bettenhauser M, Nguyen T, Ruiz J, et al. (2010) Identification of the pro-apoptotic effector domain in human Sox4. Biochem Biophys Res Commun 386: 212-217.

52. Friesen SD, Bettenhauser M, Nguyen T, Ruiz J, et al. (2010) Identification of the pro-apoptotic effector domain in human Sox4. Biochem Biophys Res Commun 386: 212-217.

51. Agaisse H, Burrack LS, Philips JA, Rubin EJ, Perrimon N, et al. (2005) Genome-wide RNAi analysis of growth and viability in Drosophila cells. Science 303: 618-619.

50. Arai K, Yamaoka Y, Yamasaki M, Nakagome M, et al. (2008) Functional characterization. Cancer Res 68: 1015-1025.

49. Ress C, Holtmann M, Maas U, Sofsky J, Dorn A (2000) 20-Hydroxyecdysone-induced differentiation and apoptosis in the Drosophila cell line, l(2)mbn. Tissue Cell 32: 464-477.

48. Cherry S, Doukas T, Armknecht S, Whelan S, Wang H, et al. (2005) Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to the actin cytoskeleton. J Biol Chem 280: 10134-10138.

47. Bjorklund M, Taipale M, Varjosalo M, Saharinen J, Lahdenpera J, et al. (2006) Discovery of JAK/STAT signalling components by genome-wide RNAi screens. PLoS Biol 4: e379.

46. Sparkes AC, Mumford KL, Patel UA, Newbury SF, Crane-Robinson C (2001) Characterization of an SRY-like gene, DSox14, from Drosophila. Gene 272: 1008-1010.

45. Eggert US, Kiger AA, Richter C, Perlman ZE, Perrimon N, et al. (2004) Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. PLoS Biol 2: e58.

44. Mills K, Daish T, Kumar S (2005) The function of the Drosophila caspase DRONC in cell death and development. Cell Cycle 4: 744-746.

43. Lehmann M, Jiang C, Ip YT, Thummel CS (2004) A balance between the diap1 death inhibitor and programmed cell death in Drosophila development. Cell Death Differ 11: 916-923.

42. Ress C, Holtmann M, Maas U, Sofsky J, Dorn A (2000) 20-Hydroxyecdysone-induced differentiation and apoptosis in the Drosophila cell line, l(2)mbn. Tissue Cell 32: 464-477.

41. Gateff E (1978) Malignant neoplasms of genetic origin in Drosophila melanogaster. Science 200: 1488-1489.

40. Bettencourt-Dias M, Giet R, Sinka R, Mazumdar A, Lock WG, et al. (2004) Functional characterization. Cancer Res 66: 3434-3442.

39. Yin VP, Thummel CS (2004) A balance between the diap1 death inhibitor and programmed cell death in Drosophila. Cell Cycle 3: 1211-1219.

38. Coulombe P, Blanpain C, Lewis J, Nelson RS, et al. (2009) Identification of the pro-apoptotic effector domain in human Sox4. Biochem Biophys Res Commun 386: 212-217.

37. Ma Y, Creanga A, Lum L, Beachy PA (2006) Prevalence of off-target effects in individual tissues by the GAL4 enhancer trap technique. Genome Res 16: 429-436.

36. Echeverri CJ, Perrimon N (2006) High-throughput RNAi screening in cultured cells: a user’s guide. Nat Rev Genet 7: 373-384.

35. Leulier F, Ribeiro PS, Palmer E, Tenes Y, Takahashi K, et al. (2006) Systematic in vivo RNAi analysis of putative components of the Drosophila cell death machinery. Cell Death Differ 13: 1663-1674.

34. Mills K, Daish T, Kumar S (2005) The function of the Drosophila caspase DRONC in cell death and development. Cell Cycle 4: 744-746.

33. Lehmann M, Jiang C, Ip YT, Thummel CS (2004) A balance between the diap1 death inhibitor and programmed cell death in Drosophila development. Cell Death Differ 11: 916-923.

32. Strober W (1997) Trypan Blue Exclusion Test of Cell Viability. In: Coligan JE, Shehu S, Beck R, McMichael AJ, editors. Current Protocols in Immunology. John Wiley & Sons, Inc.

31. Ress C, Holtmann M, Maas U, Sofsky J, Dorn A (2000) 20-Hydroxyecdysone-induced differentiation and apoptosis in the Drosophila cell line, l(2)mbn. Tissue Cell 32: 464-477.

30. Acevedo F, Fernández R, Chu P, Jolliet G, et al. (2006) Autophagy occurs upstream or parallel to the apoptosis during histolitic cell death. Development 133: 1457-1465.

29. Juliano, S., Sass, M., and Witten, E. (2005) Hid can induce, but is not required for autophagy in polyplid larval Drosophila tissues. Eur J Cell Biol 84: 541-552.