THE GENETICS OF CELL DEATH: APPROACHES, INSIGHTS AND OPPORTUNITIES IN *DROSOPHILA*

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Abstract | Cell death is ubiquitous in metazoans and involves the action of an evolutionarily conserved process known as programmed cell death or apoptosis. In *Drosophila melanogaster*, it is now uniquely possible to screen for genes that determine the fate — life or death — of any cell or population of cells during development and in the adult. This review describes these genetic approaches and the key insights into cell-death mechanisms that have been obtained, as well as the outstanding questions that these techniques can help to answer.

RNAi-MEDIATED KNOCKDOWN
A phenomenon in which the expression of a gene is temporarily inhibited ('knocked down') when a complementary dsRNA molecule is introduced into the organism.

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Cell death is ubiquitous in animals, both during development and in the adult. Much of this death shows characteristics of apoptosis, a form of cell death originally defined on the basis of the recognition that many dying cells undergo a stereotypical series of morphological changes, followed by phagocytosis by neighbouring cells or specialized phagocytes[1]. During development, the role of apoptosis is to sculpt structures, remove excess cells and delete tissues that have outlived their usefulness. Apoptosis also has an important cellular proofreading function during development: it is used to eliminate gametes and other embryonic cells with damaged DNA or abnormal chromosome content, as well as cells that are in inappropriate locations[2–4]. In the adult, apoptosis is important for tissue-size homeostasis, for balancing proliferation with cell death and for selection of the immune repertoire. Apoptosis is also important in defence against potentially dangerous cells: cells that are infected with viruses or other pathogens, cells with DNA damage and cells that are proliferating inappropriately[5–8]. Not surprisingly, deregulation of cell death has severe consequences for the developing organism and adult. Inappropriate cell death is associated with degenerative neurological diseases, stroke, cardiac ischaemia and immune suppression associated with AIDS, whereas suppression of naturally occurring cell death contributes to autoimmune disease and cancer[5,9–13].

Drosophila melanogaster is a useful system in which to study apoptosis because it occurs throughout the fly life cycle and in response to a number of insults that are relevant to human disease[4,12–15]. In addition, where it has been studied, cell death in flies and mammals uses similar machinery and mechanisms of regulation[16,17]. These observations confirm our belief in the universality of mechanisms that control cell survival and death. They also provide a firm intellectual foundation for a growing body of work that aims to use *D. melanogaster* to model human neurodegenerative diseases (reviewed in REFS 12,13). In a more applied context, the design of insect control agents to combat agricultural pests and disease vectors through the selective promotion of apoptosis or generation of sterile flies will also benefit from this work[18].

Many genetic tools are available in *D. melanogaster*. These include tools for gene targeting, a growing collection of fly lines containing single-gene disruptions and the ability to drive tissue-specific gene expression or carry out RNAi-MEDIATED KNOCKDOWN with a high degree of spatial and temporal control. In addition, the development of sophisticated genetic screens makes it possible to search for genes that participate in particular signal-transduction pathways or biological processes in essentially any cell type (reviewed in REFS 19–21). These tools have recently been used to identify new protein-encoding genes required for cell viability[22], as well as

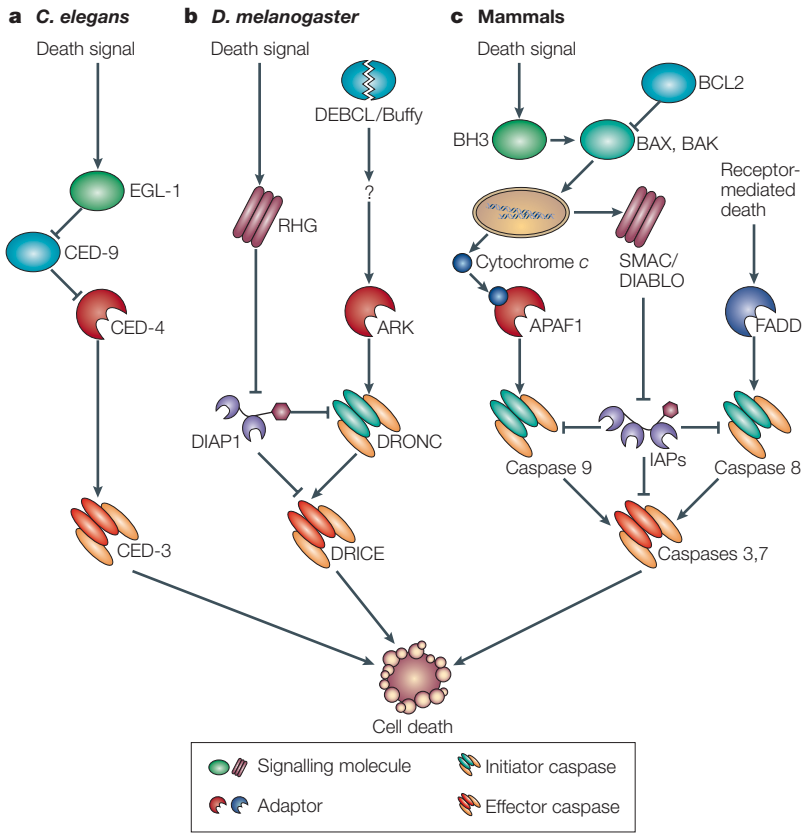

Figure 1 | **The core apoptosis machine compared in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals. a** | In *C. elegans*, the adaptor protein CED-4 promotes the activation of the caspase CED-3. CED-4 activity is inhibited by the Bcl2 family member, CED-9. Various stimuli promote death by inducing tissue-specific expression of EGL-1, which disrupts CED-9 function. **b** | In *D. melanogaster*, the adaptor protein ARK (homologous to CED-4 in worms and APAF1 in mammals) promotes activation of the apical caspase DRONC in many cells that should normally live. This activation might be regulated by the pro- and anti-apoptotic multidomain Bcl2 family members DEBCL and Buffy, but this is largely speculative (indicated by the question mark associated with the arrow). DIAP1, an inhibitor of apoptosis protein (IAP), inhibits Dronc and the effector caspases activated by DRONC, such as DRICE. DIAP1-binding proteins such as RPR, HID, GRIM, SKL and JAFRAC2 (indicated by 'RHG') partly promote death by disrupting 'the anti-caspase' function of DIAP1. **c** | In mammals, APAF1-dependent activation of caspase 9 (functionally homologous to DRONC in flies), is highly regulated by Bcl2 family proteins. Anti-apoptotic multidomain proteins are represented by Bcl2, and pro-apoptotic multidomain proteins by BAX and BAK. Many death stimuli promote the expression or activation of BH3-only family members, which facilitate BAX- and BAK-dependent release of pro-apoptotic, mitochondrially localized proteins; among these proteins are cytochrome *c*, which promotes APAF1 activity, and the IAP-binding protein SMAC/DIABLO, which disrupts the anti-caspase activity of IAPs. In a separate pathway, ligand-bound death receptors recruit adaptors such as FADD, which then recruits and activates apical caspases such as caspase 8. In both pathways, apical caspase activation leads to cleavage and activation of downstream caspases such as caspase 3 and caspase 7. Also, in both pathways, IAPs inhibit active caspases.

genes that encode microRNA (miRNA) inhibitors of cell-death[23,24]. The screens that led to the identification of these molecules did not proceed to SATURATION, making it almost certain that more components remain to be identified. Which of these molecules are important components of cell-death control mechanisms, and the contexts in which they function, are largely unknown. Proteins and miRNAs that coordinately regulate proliferation or fat metabolism and apoptosis have also recently been identified[23–30], as have non-apoptotic roles for core components of the cell-death execution machinery[31–34]. Together, these observations emphasize the fact that the regulation of cell survival always occurs in a specific cellular context, and that the outcome of miRNA or protein expression involved in the cell-death pathway depends on a number of factors — including cell-cycle and differentiation state, cell type, interacting RNAs and proteins, and signals from neighbouring cells — that are just beginning to be explored.

In this review, we first describe the core of the apoptotic cell-death machinery in *D. melanogaster* and compare it with that of worms and mammals. This brief overview is not meant to be exhaustive, but to introduce the reader to the common logic that underlies cell-death signalling across species. More detailed discussions of the cell-death machinery in individual species can be found in other reviews[16,17,35–37]. We then highlight several areas of cell-death research in which there are outstanding questions, thereby providing opportunities for further research in flies. With this as a background, we then describe the various genetic approaches that have been or could be used to identify regulators of cell death.

Caspases: the core of the cell-death machine

In flies and vertebrates (and probably in worms as well), most, if not all cells can undergo apoptosis in the absence of new gene expression, indicating that the components required to carry out apoptosis are present and ready for activation[38,39]. The core of the cell-death machinery consists of members of a family of proteases known as caspases[35], which become activated in response to different death signals. Active caspases then cleave various different cellular substrates that ultimately lead to cell death and corpse phagocytosis. Most cells, if not all, constitutively express caspase zymogens (inactive precursors) that are sufficient to bring about apoptosis. So, the key to cell death and survival signalling is in controlling the levels of active caspases in the cell. As discussed in this review, several basic strategies are used to regulate caspase activity, and the core proteins that drive caspase-dependent death are evolutionarily conserved. Interestingly, however, different organisms seem to emphasize distinct points of control. It is unknown whether these distinctions reflect chance events in evolution or specific selective pressures that have been acting on these organisms.

***Cell death in* Caenorhabditis elegans.** In worms, the sole cell-death caspase, cell-death abnormality-3 (CED-3), and its activator, the adaptor protein CED-4, are present ubiquitously. No inhibitors of activated CED-3 have been identified. However, most cells are protected from death by the expression of CED-9 (an anti-apoptotic, multidomain BCL2-family protein), which inhibits CED-4-dependent CED-3 activation. In many cells that are chosen to die, a small pro-apoptotic EGL-1 protein (of the BH3-only BCL2 family) is expressed and disrupts interactions between CED-9 and CED-4, thereby allowing CED-4 to promote CED-3 activation

SATURATION
A screen that is designed to induce at least one mutation in every gene is said to have been carried out to saturation.

(reviewed in REF. 40,41). Therefore, in this system, the decision to activate caspase-dependent cell death is made at an upstream level, through inhibition of CED-9, an inhibitor of caspase activation (FIG. 1a).

Cell death in mammals. In mammals, the primary decision to activate caspase-dependent cell death is usually made at the level of positive death signals; these signals activate so-called 'initiator' caspases, which promote apoptosis by cleaving and thereby activating short, prodomain 'effector' caspases[36]. These latter proteases mediate cell destruction by cleaving a range of substrates[42]. Caspase activity in mammals is negatively regulated at several levels. Most importantly for the purposes of this review, the activity of activated caspases is dampened through several mechanisms by the inhibitor of apoptosis (IAP) family of proteins[43,44] (FIG. 1c).

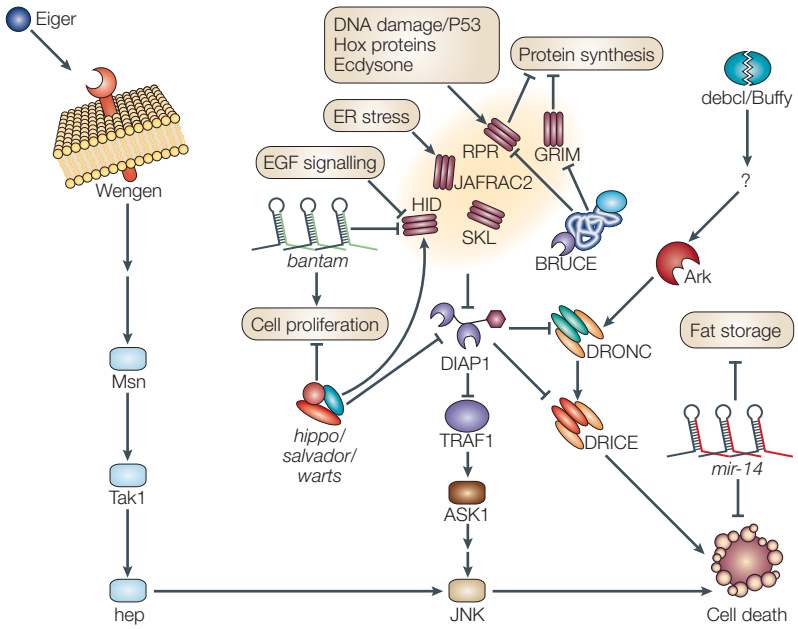

Figure 2 | **Regulation of cell death in *Drosophila melanogaster*.** A more detailed illustration of several cell-death pathways. The TNF-family ligand Eiger binds the receptor Wengen in physiological contexts that remain to be identified. This leads, through ill-defined steps (the two arrows), to activation of Misshapen (MSN), the *c*-Jun N-terminal kinase (JNK) kinase kinase kinase (JNKKKK). MSN phosphorylates and activates TAK1 (the JNKKK), which promotes activation of Hemipterous (HEP; the JNKK). HEP phosphorylates and activates JNK. In a second pathway leading to JNK activation, binding of Reaper (RPR) to DIAP1 (not shown) results in stabilization of the tumour-necrosis factor-associated factor 1 (TRAF1), because DIAP normally destabilizes it; this leads to the activation of the apoptosis signal-regulating kinase 1 (ASK1) and to JNK activation. JNK activation promotes cell death in some, but by no means all contexts. Members of the RHG family of DIAP1-binding proteins (orange cloud), are regulated through several pathways. RPR expression is activated in most, if not all, dying cells in the embryo, and is also induced by various stimuli. JAFRAC2 is released from the endoplasmic reticulum (ER) in response to UV irradiation (ER stress). HID is negatively regulated by the epidermal growth factor (EGF) receptor/Ras/MAPKinase pathway through phosphorylation (EGF signalling). The *bantam* miRNA negatively regulates HID translation, whereas *hippo/salvador/warts* stimulate HID expression. All RHG family members bind to DIAP1 and inhibit its anti-apoptotic activities. In addition, at least RPR and GRIM also have DIAP1-independent pro-apoptotic activities, one of which is the general inhibition of translation. The *mir-14* miRNA inhibits cell death and fat storage through unknown mechanisms.

***Cell death in* Drosophila melanogaster.** In contrast to the previously mentioned systems, in which caspase activation serves as the main point of control, in *D. melanogaster*, many cells experience chronic activation of the apical cell-death caspase DRONC (encoded by *NC*) mediated by the adaptor ARK, the fly homologue of CED-4 in worms and APAF1 in mammals homologue (REFS 45–49) (see FIG. 1b). If unrestrained, active DRONC cleaves and activates downstream effector caspases that bring about cell death. Cells survive because they express DIAP1 (encoded by *thread* (*th*))[39,50]; this IAP suppresses DRONC activity, as well as that of the caspases that are activated by DRONC (reviewed in REF. 51). In one important pathway, caspase-dependent cell death is induced by regulating the expression of pro-apoptotic proteins such as RPR (Reaper), HID (Head involution defective, also known as Wrinkled (W)), GRIM, SKL (Sickle) and JAFRAC2 (the RHG proteins). These proteins disrupt DIAP1–caspase interactions through several mechanisms, each of which has the effect of unleashing a cascade of apoptosis-inducing caspase activity (reviewed in REF. 52). Binding of RHG proteins to DIAP1 can also disrupt interactions between DIAP1 and TRAF1, thereby leading to activation of Jun amino-terminal kinase (JNK)[53]. JNK, which can also be activated through a TNF-like receptor-mediated pathway (of unknown function), promotes cell death in flies in some contexts (reviewed in REF. 54) (FIG. 2). Proteins that perform analogous anti-IAP functions are present in mammals (FIG. 1c), but not in *C. elegans*, which, as noted previously, lacks identified caspase inhibitors.

To summarize, in flies, DIAP1 defines an important site at which different death signals are integrated (dependent on the relative levels and activities of RHG proteins, DIAP1 and pro-apoptotic DIAP1-interacting proteins), and at which an output — caspase activity — is determined. By contrast, mammalian IAPs seem to have more of a role in modulating cell death. In this system, the analogous life or death calculation is often made at the level of interactions between pro- and anti-apoptotic members of the Bcl2 family, which regulate the release of pro-apoptotic proteins from mitochondria. As discussed in subsequent sections, the roles of Bcl2 proteins in *D. melanogaster* are just beginning to be described.

Outstanding questions and opportunities in flies. The *D. melanogaster* cell-death mechanism outlined previously highlights the principal components and features of the canonical fly apoptosis machinery, and identifies several key points of regulation *in vivo* — the transcriptional or post-transcriptional activation of several RPR-, HID- and GRIM-like proteins and their effects on IAP family members. However, many questions remain to be addressed. For example, how do these components interact with one another and regulate each other's activity? RPR, GRIM, and probably SKL, also have pro-apoptotic activities that are independent of interactions with DIAP1 (REFS 55–58). Nothing is known about these pathways. Also, what is the nature of

BACULOVIRUS CASPASE INHIBITOR P35
Baculoviruses are large DNA viruses that infect arthropods. p35 is a baculovirus-encoded protein that inhibits cell death by acting as a suicide substrate for many caspases.

RNA INTERFERENCE (RNAi)
A form of post-transcriptional gene silencing, in which dsRNA induces degradation of the homologous endogenous transcripts, mimicking the effect of the reduction, or loss, of gene activity.

EPISTASIS
When the phenotype associated with mutation of a gene (A) is masked by mutation in a second gene (B), B is said to be epistatic to A. In a switch pathway (a pathway in which the output is one of two states, often developmental fates), such an observation would indicate that genes A and B act in the same pathway, and that A acts through B.

SENSITIZED GENETIC BACKGROUND
A genetic background in which modest (twofold) changes in the dose of pathway components produce a phenotype that would not be observed in a wild-type background.

DOMINANT MODIFIER SCREEN
A signalling pathway is hyperactivated or partially deactivated in a specific tissue. These flies are often sensitive to modest changes in the levels of pathway components (heterozygosity) that would otherwise not result in a visible phenotype — but only in the specific tissue that is targeted.

NURSE CELLS
Female germline-derived cells that support the development of the oocyte. Nurse cells are interconnected to each other and to the developing oocyte through intercellular bridges that facilitate transport of RNA and protein into the growing oocyte.

IMAGINAL DISC
An epithelial sheet of cells that occurs as a sac-like infolding of the epithelium in the larva. Small groups of imaginal disc founder cells arise in the embryo. They continue to divide until pupation, when they differentiate into many adult structures (wings, legs, eyes, antennae and genitalia).

the apical signals that drive apoptotic caspase activation in the many contexts in which cell death functions in the fly? Finally, there are many cases of cell death in the fly, both naturally occurring and investigator-induced (such as fly models of neurodegenerative diseases) that use pathways other than the canonical pathway described above. These remain relatively unexplored. In the subsequent sections, we highlight some of these questions in more detail.

Naturally occurring cell death: the canonical pathway. What are the control points at which cell fate — survival or death — is regulated? In many cells targeted for death, transcriptional upregulation of combinations of *rpr*, *hid*, *grim* and *skl* has an important function. However, in most but not all cases (reviewed in REFS 59,60), the identities of factors that drive expression of these or other death activators, such as DRONC, in specific cell populations are unknown. Clearly, there is much work to be done here. Genetic screens for mutants in which specific, naturally occurring cell deaths fail to occur provide a 'top–down' method to identify these genes[61]. An alternative, 'bottom–up' approach would be to identify and characterize genomic regulatory regions — transcription-factor binding sites and the factors that bind them[62,63].

A second important unanswered question concerns whether there are upstream regulators of caspase activation in *D. melanogaster*. In mammals, cytochrome *c*, which is released from mitochondria in response to different stresses (FIG. 1c), serves as an important death signal, as it promotes APAF1-dependent activation of caspase 9. By contrast, fly proteins that promote ARK-dependent activation of DRONC have not been identified. Do these regulators simply not exist, or have they just been missed? Evidence that there is a level of control upstream of or in parallel to transcriptional activation of *rpr*, *hid* and *grim* comes from the study of corpse phagocytosis. When cells die, they are phagocytosed by their neighbours or by phagocytes (reviewed in REF. 64). Embryos that lack *rpr*, *hid* and *grim*, or wild-type embryos in which effector apoptotic caspases have been inhibited by expression of the BACULOVIRUS CASPASE INHIBITOR P35, show a pattern of cell phagocytosis similar to that observed in wild-type embryos[65]. This indicates that many cells targeted for death experience a previously unrecognized *rpr*-, *hid*-, *grim*- and caspase-independent signal that targets them for elimination. This could reflect the activity of an unidentified upstream element in the RHG–DIAP1 pathway or activation of an (unexplored) parallel pathway.

Candidates for molecules that are involved in such a pathway (in either model) are members of the Bcl2 family of proteins. In mammals, interactions between pro- and anti-apoptotic multidomain Bcl2 proteins and BH3-only pro-apoptotic Bcl2 proteins, which are each activated in response to specific environmental signals, control the release of cytochrome *c* and other pro-apoptotic factors, some of which promote death through caspase-independent mechanisms (reviewed in REF. 36). *D. melanogaster* has two multidomain Bcl2 family members, encoded by *debcl* (also known as *drob1*, *dborg1* or *dbok*) and *Buffy* (also known as *dborg2*); these proteins have pro- and anti-apoptotic activity, respectively, making them good candidates for upstream regulators of caspase activation (reviewed in REF. 66). Fly BH3-only proteins have not been identified. RNA INTERFERENCE (RNAi) mediated knockdown of *debcl* results in a decrease in naturally occurring cell death in the embryo. In addition, *Buffy* expression suppresses *debcl*-dependent death, as well as that owing to loss of DIAP1 in the embryo. These observations are consistent with models in which DEBCL and BUFFY participate in the canonical pathway (reviewed in REF. 66). Curiously, however, mutations in *debcl* and *Buffy* have not been identified in the many screens that have been carried out for components of the RHG–DIAP1 pathway (FIG. 1b). In addition, at least in cells in culture, the results of RNAi-based knockdown experiments have provided no evidence of a crucial role for cytochrome *c* in caspase activation[45,48]. One possibility is that *debcl* and *Buffy* function in the canonical pathway, but at a point that is not identified in current screens for canonical pathway components. Alternatively, they might participate in a parallel pathway. In either case, the signals that regulate their expression and activity are unknown. Mutants that disrupt these genes would be useful for addressing these questions, because they would allow the determination of EPISTATIC relationships with canonical pathway components such as *rpr*, *hid*, *grim* and *th*. Mutants would also indicate the tissues in which these genes are required in the fly. This would provide the information necessary to construct SENSITIZED GENETIC BACKGROUNDS (created by RNAi-driven, tissue-specific, partial loss-of-function phenotypes) that would facilitate screens for interacting components (see later section on DOMINANT MODIFIER SCREENING).

Naturally occurring cell death: evidence for non-canonical pathways. As noted above, not all naturally occurring cell deaths require the activation of the canonical pathway. Four examples from *D. melanogaster* illustrate this point.

During the late stages of oogenesis, germline-derived NURSE CELLS transfer the bulk of their cytoplasm to the growing oocyte in a process called 'dumping'. Shortly after, the nurse cells die, showing many features of apoptosis (reviewed in REF. 67). Dumping and apoptosis might be linked because, in some mutants in which transfer of the nurse-cell cytoplasm does not happen, nurse cells do not undergo apoptosis. Analysis of hypomorphic or null mutants indicates that this death requires the cell-cycle regulators E2F and DP1, and the zinc-finger protein-encoding gene *pita*, but does not require *rpr*, *hid*, *grim* or the caspase *Dcp1*. Nurse-cell death is also insensitive to overexpression of DIAP1 (reviewed in REF. 67). Together, these observations support the arguement that nurse-cell death occurs through a novel, perhaps caspase-independent pathway. Loss-of-function or germline-overexpression screens for female-sterile mutants with a 'dumpless' phenotype provide one approach to identifying regulators of this process.

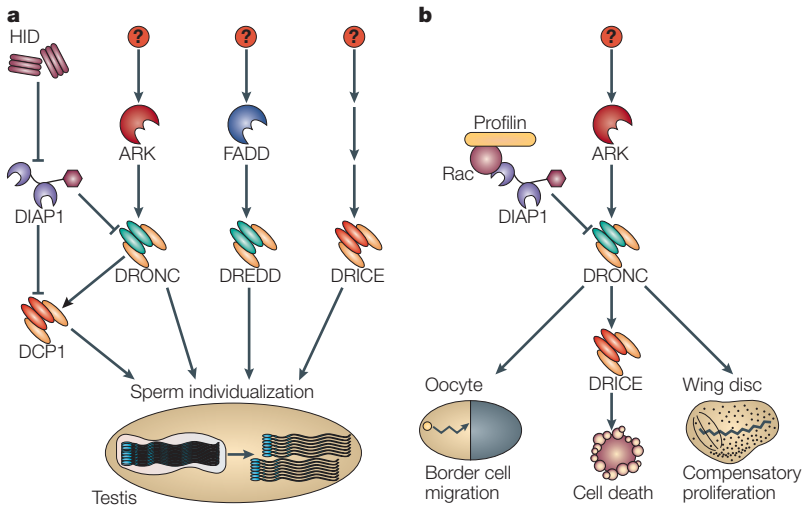

Figure 3 | **Non-apoptotic roles of apoptotic caspases in *Drosophila melanogaster*.** **a** | During the late stages of spermatogenesis spermatids, which develop within a SYNCYTIUM, must become separated from each other. This individualization involves the activity of many caspase cascades, including those that culminate in the activation of DRONC and DCP1, DREDD and DRICE[32]. The mechanisms by which these cascades are activated, and their targets, are unknown. **b** | During the migration of somatic follicle cells, known as border cells, Profilin and Rac interact with DIAP1, which inhibits DRONC activity. DRONC participates in border cell migration[34]. When cells in the fly wing-disc die as a result of stress (induced by heat or X-ray irradiation), they are replaced by neighbouring cells, which undergo compensatory proliferation. This helps to maintain a constant tissue size. DRONC activity is required for compensatory proliferation, although where and how DRONC acts is unknown[33].

Massive amounts of larval tissue are destroyed and replaced by cells derived from IMAGINAL DISCS during metamorphosis. Much of this death is AUTOPHAGIC, rather than apoptotic in morphology (reviewed in REF. 68). Canonical pathway components (caspases, Rpr, HID and DIAP1) participate in these deaths. However, the results of transcriptional profiling experiments indicate that tissues undergoing autophagic death express a distinct set of genes that has little overlap with that seen in cells undergoing apoptosis induced by ionizing radiation. This, together with observations that inhibition of caspases — by expression of p35 or dominant negative versions of DRONC — prevents some, but not all changes in cells targeted for autophagic cell death, provides evidence that these deaths use distinct pathways (reviewed in REF. 68).

Several dominant and recessive neurodegenerative diseases have been modelled in *D. melanogaster* (reviewed in REFS 12,13,69). Where examined, the cell deaths that take place in these diseases are at least in part caspase-independent, in that they are not inhibited by co-expression of baculovirus p35 (REFS 70,71). Dominant modifier screens, to illustrate one approach, have begun to identify regulators of these deaths (reviewed in REFS 12,13,69), but little else is known about the effector mechanisms involved.

A recent genome-scale RNAi screen in two *D. melanogaster* haemocyte cell lines identified several uncharacterized genes required for cell survival[22]. As the cell lines that have been tested only express a fraction of fly genes, it is almost certain that more of these genes exist for other cell types. Interestingly, some of the genes lack clear homologues in other species, indicating that they are unlikely to be housekeeping genes and might identify novel points of regulation. These observations, together with those already described, indicate that we have just begun to scratch the surface in terms of identifying and characterizing cell-death regulators and the contexts in which they function.

Finally, it is important to note that HID, as well as several caspases whose activation is thought to be crucial for naturally occurring cell death — DRONC, DRICE and DCP1 — also participate in non-apoptotic processes. These include SPERMATID differentiation[32], compensatory proliferation in response to ectopic cell death in imaginal discs[33] and ovarian BORDER-CELL migration[34] (FIG. 3). Caspases also have non-apoptotic roles in mammals (reviewed in REFS 72,73). The pathways that drive caspase activation in these non-apoptotic contexts, and the identities of important caspase targets, are not known. But the genes encoding these molecules can presumably be identified in the fly from mutants that have phenotypes similar to those associated with caspase inhibition in the aforementioned contexts. It is also unclear how cells that activate apoptotic caspases in non-apoptotic contexts avoid cell death. It seems probable that tight control over the site of caspase activation (or stabilization of the active caspase), or perhaps the action of novel inhibitors, will be important. Screens for mutants in which excess caspase activation and/or cell death occur during spermatid differentiation (resulting in male sterility) or border-cell migration (resulting in female sterility) provide a straightforward approach to identifying these molecules.

Forward genetic approaches

Pioneering screens in *C. elegans* identified the global regulators of cell death: the caspase CED-3, its activating adaptor CED-4 and the CED-4 inhibitor, CED-9 (reviewed in REF. 40). *Caenorhabditis elegans* was (retrospectively) a particularly advantageous organism in which to carry out such a screen because although the loss of naturally occurring cell death resulted in a visible phenotype (an absence of corpses in a sensitized background in which corpse phagocytosis failed to occur), it was not lethal. So, it was possible to identify mutations in global regulators of cell death simply by screening for viable animals that lacked cell death-associated corpses. Workers with other organisms, such as flies, zebrafish and mammals, in which cell death has many essential functions during development, are not so fortunate, and therefore demand the use of different screening strategies. Below, we describe some of the main genetic-screen-based approaches that have been, or could be, used to identify cell-death regulators in the fly.

The F_2 loss-of-function screen. One common approach is the F_2 screen (FIG. 4a). As already described for *C. elegans*, in some fortunate situations, interesting mutants show homozygous viablility. Indeed, the first cell-death mutants in flies that were identified as such, *roughest* and *echinus*, are homozygous-viable recessive mutants

SYNCYTIUM
A multinucleate cell in which the nuclei are not separated by cell membranes.

AUTOPHAGY
In autophagic cell death, as opposed to apoptotic cell death, the cell is degraded largely from within, with little or no help from phagocytes. Bulk cytoplasm and organelles are sequestered within double-membrane-bound vesicles. These ultimately fuse with the lysosome and their contents are degraded.

SPERMATID
A post-meiotic haploid male germ cell.

BORDER CELLS
A small group of specialized somatic follicle cells. They delaminate from the follicular epithelium, invade the underlying germline tissue and migrate towards the oocyte.

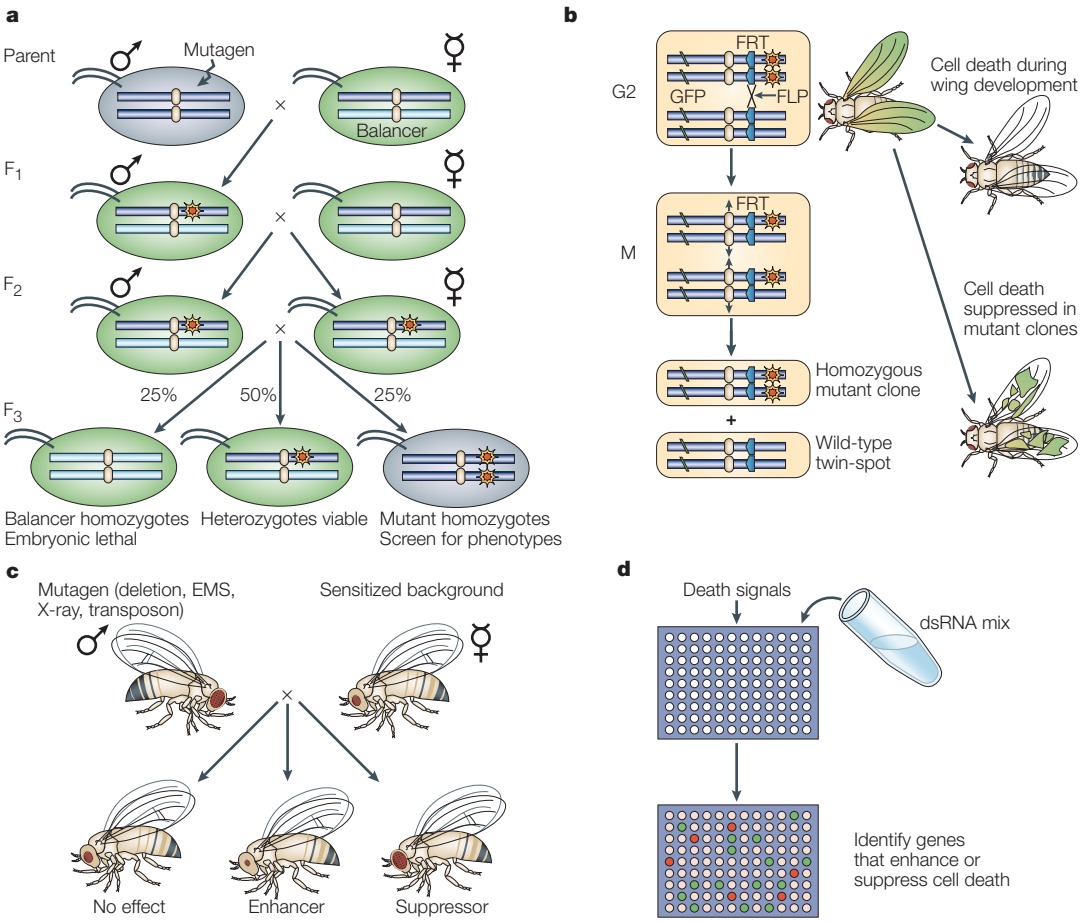

Figure 4 | **Genetic screens for cell-death regulators in *Drosophila melanogaster*. a** | In an F_2 screen, animals homozygous for a mutation (the red and yellow asterisk), generated through the series of crosses indicated, are examined for phenotypes suggestive of defects in cell death. These might include embryonic lethality or, if the homozygotes are viable, phenotypes such as defective adult structures (eyes or wings), male or female sterility or a short lifespan. A balancer chromosome (Balancer) prevents recombination with the chromosome that is being mutagenized and homozygosed. **b** | In a clone-based screen, the goal is to generate marked patches of homozygous mutant tissue in an otherwise heterozygous (and therefore viable) background. In brief, the FLP/FRT system is used to drive MITOTIC RECOMBINATION on a specific chromosome arm (the one that carries FRT sites that are targets for the FLP recombinase) in specific populations of cells (those that are mitotically active and that express the FLP recombinase). If these flies are heterozygous for a mutation (as would be the case if one of the parents were mutagenized in the previous generation), then clones of tissue homozygous for the mutation will be generated in the tissue of interest. In the flies illustrated, clones of mutant tissue are generated in the wing for a mutation that blocks normal cell death; expression of green fluorescent protein (GFP) is lost from the wing cells that die (see main text) so the adults contain patches of green (GFP positive) tissue on a dark (heterozygous or homozygous wild-type (the twin-spot)) background. **c** | In a dominant modifier screen, wild-type flies are mutagenized and crossed to flies that are in some way sensitized. In this case, they have small eyes owing to expression of a death activator in the eye. Progeny are screened directly for enhancers or suppressors, which might encode other pathway components. An important limitation of the dominant modifier screen is that only components for which activity is rate-limiting (sensitive to a two fold decrease or overexpression) in the sensitized background will be identified. Therefore, to gain a complete picture of a pathway it is often necessary to carry out many screens using flies that are sensitized at different points in a pathway (reviewed in REF. 19). **d** | In a cell-culture-based RNA interference (RNAi) screen, cells in 96-well plates are treated with dsRNA for many genes (as many as you want to test) and some cellular phenotype is assayed several days later. In the illustrated case, the cells have been treated with a mild death stimulus that causes a fraction of them to die (the pink wells). Inactivation of genes that promote cell death results in increased cell survival (green wells), whereas inactivation of genes that promote cell survival result in increased cell death (red wells). EMS, ethyl methanesulphonate; M, mitosis.

MITOTIC RECOMBINATION
A crossover between two homologous dsDNA molecules that leads to a physical exchange of DNA and genetic information. This recombination occurs frequently during meiosis, but is relatively rare during mitosis. As a consequence of mitotic recombination, cells can undergo a 'loss of heterozygosity' or gene conversion.

BALANCED STOCK
A stock that carries a lethal mutation on one chromosome homologue, and a balancer chromosome on the other. A balancer chromosome carries multiple inversions that prevent recombination with the lethal-bearing chromosome, a recessive lethal mutation and a dominant marker. Matings between balanced lethal flies produce only balanced lethal adult progeny — a stable stock.

with specific defects in naturally occurring cell death in the fly eye[74]. A more recent F_2 screen for global regulators of cell death eliminated the requirement for homozygous viability[75]. White and colleagues (fortuitously) used as their 'mutagen' a set of publicly available BALANCED STOCKS that each carried a heterozygous deletion (also known as a deficiency) for a specific region of the genome. They screened collections of living embryos from these lines with Acridine orange, a fluorescent dye that is taken up and retained by many dying cells (FIG. 5a,b). Using this collection, they were able to test 60–70% of the genome for global cell-death activators

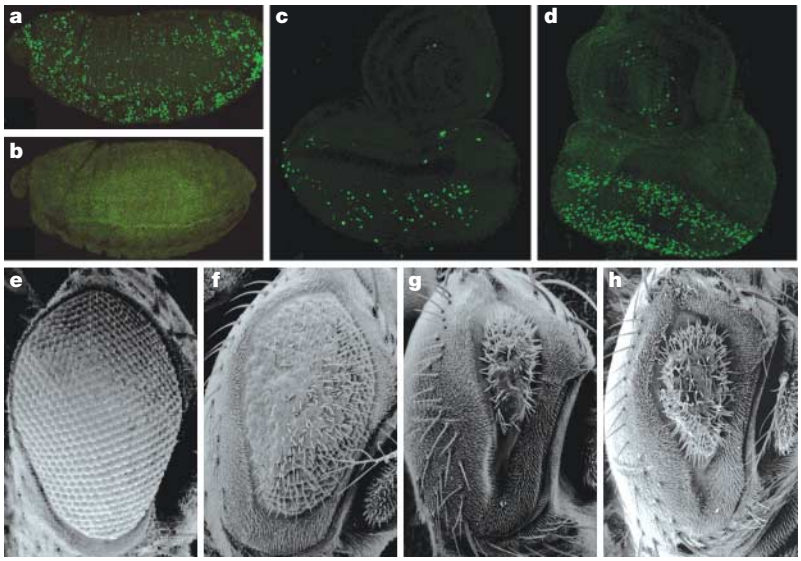

Figure 5 | **Cell death phenotypes in *Drosophila melanogaster*. a**,**b** | Wild-type (**a**) and H99 homozygous (**b**) embryos stained with the dye Acridine orange. The H99 deletion removes *reaper* (*rpr*), *head involution defective* (*hid*) and *grim*. Wild-type embryos show high levels of cell death (green spots), whereas H99 embryos show no specific Acridine orange staining. **c** | An eye imaginal disc from a wild-type third-instar larva stained with Acridine orange shows low levels of death. **d** | Eye discs from flies that express GMR-*rpr* (GMR stands for glass multimer reporter) show high levels of death in the regions of the disc in which the GMR promoter drives gene expression. **e**–**h** | Wild-type adult fly eyes show a crystalline array of roughly 800 ommatidia (eye units; **e**). Expression of GMR-*rpr* results in increased cell death and a smaller eye (**f**). This phenotype is enhanced when one copy of *diap1* (as well as many other genes) is removed using the deficiency chromosome *TH117* (**g**). A similar phenotype is seen when GMR-*rpr* flies are heterozygous for *th5*, a point mutation in the *diap1* coding region (**h**). Panels **e**–**h** reproduced with permission from REF. 50 © (1995) Elsevier Science.

(the loss of which should result in a decrease in cell death) in only ~200 crosses. Deletions of the 75C region of the fly genome resulted in the loss of essentially all naturally occurring cell death[75]. This region was subsequently found to contain three pro-apoptotic genes, *rpr*, *hid* and *grim*, each of which contributed to the deficiency phenotype[75–77]. So, a deficiency screen for global cell-death regulators succeeded where a traditional F_2 screen for single-gene mutations would have failed, as mutations that eliminate *rpr*[78] or *hid*[76] alone still have significant levels of naturally occurring cell death.

There has recently been a notable increase in the quantity and quality of the tools available for carrying out deficiency and single gene disruption F_2 screens (and other screens, as detailed below). It is now possible to create designer deficiencies with molecularly defined breakpoints. Collections of these deficiencies, generated in an ISOGENIC genetic background, are becoming publicly available, as are lines of flies that carry transposon insertions in or near more than 50% of annotated genes (reviewed in REF. 79). This collection, in conjunction with preexisting collections of single-gene mutations generated using chemicals or radiation, greatly facilitates the identification and cloning of interesting genes in the identified intervals (see the discussion of the cloning of DIAP1 below).

These new F_2 screening tools will benefit several studies of cell death in the embryo. As previously discussed, zygotic expression of *rpr*, *hid* and *grim*, and of *th* are globally required to promote naturally occurring cell death and cell survival, respectively, in the embryo. The screen that led to the identification of the RHG region examined only a fraction of the genome, and no screens have been carried out for genes, such as *diap1*, that are required globally for cell survival in the embryo. Similarly, no screens have been carried out for genes that promote or prevent cell death induced in the embryo by environmental stresses such as UV irradiation (a screen for regulators of UV-dependent death in the eye has been carried out[80]) or hypoxia. Particularly with respect to hypoxia, *D. melanogaster* embryos are fairly unusual. Embryos can survive days in the absence of oxygen and resume normal development when oxygen is restored[81]. This contrasts with the rapid cell death that occurs when mammalian cells such as neurons or cardiomyocytes are similarly deprived[9]. The question of how hypoxia resistance in flies is brought about can be posed in many ways (REF. 82). One of these is: why don't these cells die? A simple screen for homozygous embryos that are hypoxia sensitive (such as a deficiency screen similar to that which led to the identification of *rpr*, *hid* and *grim*) could provide some answers.

Tissue-specific loss-of-function screens. It is also possible to carry out tissue-specific loss-of-function screens for death regulators using a clone-based screening approach (FIG. 4b). This strategy has been used to identify genes that negatively regulate cellular growth, by scoring for a clone-overgrowth phenotype. Of particular interest for the purposes of this review are screens that identified mutations in three evolutionarily conserved genes, *hippo* (*hpo*[25–27,29], also known as *dMST*[28]), *salvador* (*sav*; REF. 83, also known as *shar-pei* (*shrp*; REF. 84)) and *warts* (*wts*; REF. 85, also known as *lats*; REF. 86). Loss of any of these genes results in a marked overgrowth of clone tissue. These genes normally function (at least in part together) in a similar way to tumour suppressors, by restricting proliferation and promoting death. They promote death — through mechanisms that remain to be described —partly by downregulating DIAP1 levels and upregulating HID transcription (reviewed in REF. 87) (FIG. 2).

Clone-based screens that are focused specifically on identifying cell-death regulators have not been carried out. However, they easily could be. The key, as in all genetic screens, is the ability to rapidly identify the phenotype of interest — cells in which cell-death signalling is disrupted. The adult wing provides an example of how this could be done. Epidermal cells that make up the *D. melanogaster* wing — can be visualized in living animals that express a nuclearly localized green fluorescent protein (GFP) in cells of the wing. GFP staining in wing-epidermal cells of wild-type animals is normally lost after about 2 hours. However, it remains when cell death is prevented by caspase inhibition[88]. This phenotype provides an ideal readout for a clone-based screen for cell-death activators (FIG. 4b).

Clone-based screens can also be carried out in tissues in which the phenotype scored for is (initially) an indirect

ISOGENIC
Cells or organisms that are derived from the same parent and therefore have almost identical genomes.

readout of possible defects in cell death. The ovary provides an example of the potential of this approach. As previously noted, failure of germline nurse cells to die on schedule is often associated with a dumpless phenotype, which results in female sterility (the primary screen phenotype). The dumpless phenotype can be visualized directly in dissected adult ovaries (the secondary screen phenotype). Several female GERMLINE CLONE SCREENS have been carried out[89–91]. In addition, a large collection of lethal *P*-ELEMENT insertions on the third chromosome has been tested for effects on oocyte differentiation through the generation of germline clones[92]. In this screen, oocyte development was disrupted in about 10% of the lines. Of these, four showed a dumpless phenotype. Although it remains to be demonstrated that any of the genes that are presumably disrupted by these *P*-element insertions normally act to promote nurse-cell death, they are clearly candidates worth testing.

Clone-based screens for cell-death inhibitors are less straightforward than those for death activators because there are more (uninteresting) ways of generating clones in which no cells survive than there are of generating clones in which there is excess survival. To identify those essential genes that are specifically required to inhibit apoptosis in a particular tissue it will be necessary to have some sort of secondary assay. For example, one would want to distinguish between mutations in genes required for cell differentiation (failure of which often leads to death through unknown mechanisms), essential cellular functions or cell proliferation, and those that are required to inhibit apoptosis. Rescue of mutant cell survival and function in the presence of a caspase inhibitor such as p35 provides one such assay. However, rescue by p35 will, by definition, only highlight mutations in death inhibitors that act upstream of, and through, caspase-dependent pathways. Other interesting pathways that do not fulfill this criterion might well be present.

The dominant modifier screen: the workhorse of the cell-death field. A third approach to identifying death regulators that has been successful is the dominant modifier screen (FIG. 4c). The power of such a screen is demonstrated by the first dominant modifier screen for cell-death regulators in *D. melanogaster* — a deficiency screen for enhancers of a small eye phenotype resulting from cell death induced by expression of *rpr* or *hid* in the fly eye (GMR-*rpr* and GMR-*hid* flies (GMR, glass multimer reporter)). Multiple deficiencies that uncovered the 72D region were identified as strong dominant enhancers in this assay. Single-gene point mutants (alleles induced using the mutagen ethyl methanesulphonate) of *th* (*th*^{ems}), mutations located in the deficiency region, gave rise to a similar enhancer phenotype, and failure of these mutants to complement a lethal *P*-element insertion in the region defined the exact location of the relevant gene, which was subsequently cloned (REF. 50). Since this early work, many dominant modifier screens for regulators of cell death have been carried out. Most of these (but not all, see REF. 93) began with a sensitized genetic background resulting from eye-specific overexpression of a cell-death activator. The eye is an ideal system for dominant modifier screens because it consists of a large, repeating array of ~800 identical eye units that is easily scored for defects in cell death under a dissecting microscope. However, any tissue in which the enhancement or suppression of death, or the consequences of these (that is, female sterility, failure to remove a GFP-labelled tissue and organismal lethality) can be scored, is amenable to this approach.

Variations on the dominant modifier screen: the gene activation screen. *Drosophila melanogaster* encodes a second IAP, DIAP2, which also acts as a strong suppressor of *rpr*- and *hid*-dependent death[50]. However, deficiencies that should eliminate DIAP2 were not identified as enhancers in the original GMR-*rpr*/GMR-*hid* screen for death suppressors described above. There are several possible reasons for this. First, a 50% decrease in DIAP2 might be compensated for by high endogenous levels of DIAP1. Alternatively, DIAP2 might simply not be expressed at significant levels in the eye, thereby making it (and any other genes not expressed in the eye) invisible in eye-based loss-of-function modifier screens. Tissue-specific gene misexpression provides a tool that bypasses some of these problems.

Gene misexpression screens use a *P*-element that carries a promoter near one *P*-element end, pointing outwards into the surrounding genomic region. When activated, this promoter can drive the expression of appropriately oriented genes in the surrounding genomic region[94,95]. Interesting lines can be identified based on modifier phenotypes in sensitized backgrounds and dominant phenotypes in a wild-type background. Both kinds of screens have led to the identification of important death regulators. One of these is *Bruce*, the *D. melanogaster* homologue of mammalian *Bruce*, which encodes a large protein with both BIR and E2 ubiquitin conjugation domains[96]. *Bruce* was identified in *D. melanogaster* as an overexpression suppressor of *rpr*- and *grim*-, but not *hid*-dependent cell death. Other examples include the E1 ubiquitinating activating enzyme (UBA1), two components of an SCF-type E3 ubiquitin ligase (*skpA* and a novel F-box gene, *morgue*) and the deubiquitinating enzyme FAF; these were identified as overexpression enhancers of death induced by GMR-driven expression of a *grim/rpr* protein fusion[97] (several of these genes were also identified in other loss-of-function modifier screens[93,98]). The miRNA *mir-14* was also identified as an overexpression suppressor of *rpr*- and *grim*-dependent cell death[23]. Finally, the *bantam* miRNA was identified on the basis of a large-eye phenotype associated with eye-specific expression in a wild-type background[24,99]. Characterization of the basis for this phenotype, as with *hpo*, *sav* and *wts*, revealed that it was due not only to increased proliferation, but also to decreased cell death, in this case owing to translational inhibition of *hid* expression[24].

What is the future of dominant modifier and/or overexpression screens for cell-death regulators in *D. melanogaster*? So far, almost all screens for death regulators have used the eye as a screening system. These

GERMLINE CLONE SCREEN
A genetic screen in which clones of homozygous-mutant germline tissue are produced in adult females. Oocytes and eggs derived from these clones (which can be distinguished from those derived from heterozygous germline tissue in several ways) can be examined for phenotypes during oogenesis and embryogenesis.

P-ELEMENT
A member of a family of transposable elements that are widely used as the basis of tools for mutating and manipulating the genome of *Drosophila melanogaster*.

CELL MICROARRAYS
Cells are plated directly onto a slide containing thousands of microarrayed spots of DNA. Cells landing on these spots are transfected with the arrayed plasmids, and can then be scored in various assays.

screens will miss death regulators that only function in specific contexts (that is, tissue-specific regulators of cell death). In addition, almost all dominant modifier screens for cell death that have been carried out until now have used sensitized systems based on gene overexpression (for example, of *rpr*, *hid*, *grim* or of proteins associated with human neurodegenerative disease). Many human diseases that involve deregulation of cell death are recessive; these include various forms of retinal degeneration, muscular dystrophies, spinal muscular atrophy and Parkinson disease (see the OMIM database for a complete list of human diseases with a genetic component). Historically, these have been difficult to model, either because fly mutations are not available or because homozygous mutants are sick or die. However, as discussed in the next section, it is now possible to create tissue-specific partial loss-of-function phenotypes for essentially any gene, at any developmental stage, using transgene-driven, tissue-specific RNAi. Therefore, disease-associated genotypes (and therefore phenotypes) can now be modelled in specific tissues, providing the substrates for future screens.

Reverse genetic approaches

General techniques for carrying out targeted gene inactivation in flies have, until recently, remained elusive. This has severely limited the usefulness of *D. melanogaster* for the systematic alteration of specific genes and analysis of the consequences. This obstacle has now been removed by the development of two technologies, targeted gene replacement and RNAi (reviewed in REFS 20,100,101). Of the two techniques, RNAi is the one that has had the most influence on genetic analysis in flies by allowing screens to be carried out in a high-throughput manner. The phenotype-based, forward genetic screens provide ways of identifying regulators of cell death in intact animals, without a specific theory in mind, other than that these deaths are under genetic control. Genome-scale RNAi screens[22,102] (FIG. 4d) now provide a way to carry out similar experiments in cell culture.

Cell-culture-based RNAi screens. RNAi-based screens (loss-of-function) that are carried out in a single cell type (either *in vivo* or *in vitro*) are limited in ways similar to those of the dominant modifier screens — not all genes of potential interest will be expressed in any one cell type. However, it is now also possible to carry out genome-scale overexpression screens in cell culture. These make use of CELL MICROARRAYS (reviewed in REF. 103). The main limitation holding back the field of cell-culture-based screens in *D. melanogaster* is the fact that there is no technology for rapidly generating cell lines from defined cell types with defined genetic backgrounds. Therefore, cell-culture-based screens for death regulators in *D. melanogaster* can be carried out in only a limited number of cellular and genetic contexts (reviewed in REF. 104). These points notwithstanding, cell-culture-based screens have distinct advantages over traditional animal-based screens, for many purposes. First, every gene is tested in a finite number of samples (assuming that all genes have been identified, something that, at this point, is probably not true). This is in contrast to random mutagenesis, in which the number of mutants screened must be many times more than that of the total number of genes in the genome to have some confidence that every gene has been hit (usually this is incorrect, as there are still mutagen-specific biases). Second, because each gene screened in an RNAi library has already been sequenced, its identity is immediately known.

One recent example, presented in BOX 1, describes a broad-based, genome-scale screen that should identify many genes required for cell survival — in the cell types tested, under the specific test conditions. More focused RNAi screens designed to identify components of specific caspase pathways can also be carried out. For example, genes required to mediate or suppress cell death in response to loss of DIAP1 could be identified as suppressors or enhancers, respectively, of RNAi directed against DIAP1. By a similar logic, genes that regulate survival in response to environmental stimuli (unfolded proteins, DNA damage and free radicals) could be identified as enhancers and suppressors of death induced by treatment of cells with reagents that induce these stresses. In addition, cells that have been engineered to function as reporters for activation or inhibition of specific pathways can be used. For example, the apical caspase DREDD, originally isolated as a potential inducer of apoptosis that functions downstream of *rpr*[105], is now recognized for its essential role in activating the innate immune response following infection by Gram-negative bacteria. In this pathway, the bacterial-wall-derived lipopolysaccharide binds to cell-surface receptors of the PGRP (peptidoglycan recognition protein) family, which

Box 1 | A cell-culture-based RNAi screen for cell survival proteins

Boutros and colleagues[22] treated two different blood-cell lines, KC cells and S2 cells, with 19,470 dsRNAs corresponding to ~91% of *Drosophila melanogaster* genes. Cells were characterized 5 days later for total ATP levels, which provide a gross measure of cell viability, metabolism and/or proliferation. Four hundred and thirty-eight dsRNAs were identified that caused a significant decrease (greater than three standard deviations) in ATP levels. RNA interference (RNAi) of 22 of these genes resulted in a decrease in ATP levels in one or both cell lines comparable to that associated with RNAi of DIAP1, which rapidly induces apoptosis in both cell types. Interestingly, although some of these genes have known or probable functions based on homology (for example, transcription factors, ubiquitin), a number of genes lack clear homologues and contain no recognizable domains. Follow-up assays are needed for most of these genes to determine which are required for cell survival, as opposed to growth and/or proliferation. That said, it is worth pointing out that the fact that some of these proteins appear to be *D. melanogaster*- or insect-specific does not make them uninteresting in the context of identifying evolutionarily conserved pathways that regulate death. True cellular homologues of *rpr*, *hid* and *grim* have not been identified outside insects. However, it was the characterization of these proteins — the identification that their N-termini were shown to be crucial for cell death[111], that this domain mediates binding to DIAP1 (REF. 111) and that this binding disrupted the ability of DIAP1 to inhibit caspases[39] — that led to our current understanding of how inhibitor of apoptosis protein anti-caspase activity is negatively regulated to promote apoptosis (reviewed in REFS 43,51). Therefore, it is quite possible that study of these seemingly unique proteins will provide general mechanistic insights into ways in which cell survival is controlled.

in turn leads, through several intermediate steps, to Dredd-dependent cleavage of the transcription factor Relish. This cleavage promotes the movement of Relish to the nucleus and the transcriptional activation of genes, such as *Diptericin (Dipt)*, that encode antimicrobial peptides (reviewed in REF. 106). Foley and O'Farrell generated cell lines that expressed β-galactosidase under the control of the *Dipt* promoter[102]. These *Dipt-lacZ* cells were then screened for enhancement or suppression of *Dipt-lacZ* induction by lipopolysaccharide, or constitutive expression in the absence of lipopolysaccharide. One of the genes identified in the latter category, *Dnr1*, encodes a potential inhibitor of DREDD, thereby highlighting the ability of a reporter-based screen to identify regulators of caspase function.

Summing up and looking forward

We have provided an overview of the state of the cell-death field in *D. melanogaster* to highlight some of the unanswered questions, as well as the genetic screening tools available to further this work. Central elements of one important pathway, defined by the core components *rpr*, *hid*, *grim*, *th*, *Nc* and *ark*, have been identified and it is clear that this pathway participates in many cell-death events in the fly. Nonetheless, many questions remain about how this pathway is activated and how its components regulate each other's activity. In addition, the concerted efforts of many researchers studying normal development — embryogenesis, metamorphosis and oocyte maturation in particular — and neurodegenerative conditions induced through mutation or other experimental manipulations, have identified cell deaths that use other pathways. Many cell-death pathways are also used in mammalian cells (reviewed in REFS 107,108). This, together with the general high level of gene conservation between flies and humans (60%–70% of human disease genes have fly counterparts[109]), shows that the continued study of cell death in flies is likely to benefit our understanding of human disease. The creation of whole-genome, protein-interaction networks currently provides a popular method by which to move from known components to new molecules, and therefore hopefully to mechanistic insights. However, the fact that a recent two-hybrid-based protein interaction map in *D. melanogaster* failed to identify any of the known physical interactions between DIAP1 and RPR, HID, GRIM, DRONC, DRICE and DCP1 (REF. 110), highlights the limitations of this approach and emphasizes the importance of genetic screens, which are fundamentally function-based, as the key tool with which to move this field forward.

With the tools available in *D. melanogaster*, it is possible to carry out screens for genes that affect the fate of any cell population, in almost any genetic background. However, only a small number of screens have been carried out, in a limited number of tissues (often the eye) or environmental contexts (healthy cells in culture), and none of these screens has been carried to saturation. The fact that key apoptotic caspases, the activation of which would reliably lead to death in many cells, can also have important non-apoptotic roles highlights the point that cellular context is everything in terms of determining function and mechanisms of regulation. There is no single kind of screen that is the best. In every screen there are tradeoffs between speed, coverage, specificity in the phenotype being initially scored and ease of gene cloning that will influence an investigator's decision (see the discussion in REF. 19). In addition, no one screen has the ability to provide a complete picture of the process under study. F_2 screens for embryonic death regulators might fail to identify genes that have many functions or have a strong maternal component. Modifier screens can be insensitive to two-fold changes in the levels of components that are not rate limiting (but are essential for the process under study). In addition, clone-based screens can fail to identify genes if their activity is also required to generate clones (such as proliferation) or to generate the cells to be studied (differentiation).

In summary, much remains to be learned about how cell death is controlled in the fly, and this will require many screens. These screens have been successful in the past, and with the new tools that have become available recently, there is every reason to believe that they will continue to be so.

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