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Genetic control of programmed cell death during animal development

Barbara Conradt

Department of Genetics, Norris Cotton Cancer Center, Dartmouth Medical School, 7400 Remsen Bldg., Hanover, NH 03755, barbara.conradt@dartmouth.edu Phone: (603) 650-1206, Fax: (603) 650-1188

Abstract

The elimination by programmed cell death of ‘unwanted’ cells is a common feature of animal development. Genetic studies in the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster* and the mouse have not only revealed the molecular machineries that cause the programmed demise of specific cells, but have also allowed us to get a glimpse of the types of pathways that regulate these machineries during development. Rather than giving a broad overview of programmed cell death during development, the current review will focus on recent advances in our understanding of the regulation of specific programmed cell death events during nematode, fly and mouse development. These studies have revealed that many of the regulatory pathways involved have additional important roles in development, which confirms that the programmed cell death fate is an integral aspect of animal development.

Keywords

Apoptotic cell death; autophagic cell death; necrosis; animal development; developmental control mechanisms

INTRODUCTION

The two most common forms of programmed cell death that can be observed during animal development are apoptotic cell death and autophagic cell death (also referred to as programmed cell death types I and II, respectively), which can be distinguished at the morphological and molecular levels. The mechanisms of apoptotic and autophagic cell death have recently been reviewed elsewhere (reviews) (47,227–229). Therefore, I will only briefly summarize aspects of these mechanisms that are relevant for the discussion of the regulatory pathways that control apoptotic and autophagic cell death during animal development.

Apoptotic cell death

Apoptotic cell death is characterized by a series of morphological changes such as shrinkage of the cell, contraction of the cytoplasm, condensation of chromatin, and engulfment by phagocytic cells. At the molecular level, apoptotic cell death is characterized by the activation of ‘caspases’ (cysteiny, aspartate-specific proteases). Once fully active, these cell-death proteases induce processes that irreversibly lead to the demise of a cell. Therefore, the maturation and activation of caspases represents the point of no return in animals as diverse as

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The author is not aware of any bias that might be perceived as affecting the objectivity of this review.

the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and mammals (reviews) (47,83,84,120,194,227). For this reason, the question of how apoptotic cell death is controlled during animal development basically boils down to the question of how caspase activation is controlled (Figure 1).

Depending on the animal model, there are slight differences between the molecular mechanisms that lead to caspase activation. In nematodes, flies, and mammals, the maturation and activation of caspases is facilitated by the 'apoptosome', a scaffold for pro-caspases that allows their self-activation. Apoptosomes are composed of a single, APAF1 (APAF1, apoptosis promoting factor 1) -like adaptor protein, CED-4 in the case of *C. elegans*, Dark in the case of *D. melanogaster*, and APAF1 in the case of mammals (reviews) (47,83,84,120,194,227). In nematodes and mammals, the assembly, and hence activation, of the apoptosome is controlled by members of the superfamily of BCL2 proteins (reviews) (45,227). Members of this family come in two flavors and fall into three different subfamilies: the anti-apoptotic BCL2-like proteins, the pro-apoptotic BAX-like proteins, and the pro-apoptotic BH3-only proteins. (Anti-apoptotic BCL2-like proteins and pro-apoptotic BAX-like proteins are also referred to as 'multi-domain' BCL2-like proteins.) Specifically, in *C. elegans* the assembly of CED-4 is blocked in healthy cells through the anti-apoptotic BCL2-like CED-9 protein. In response to apoptotic stimuli, the pro-apoptotic BH3-only protein EGL-1 blocks CED-9 function, thereby allowing CED-4 assembly and the activation of the caspase CED-3 (reviews) (84,120) (Figure 1). In mammals, the assembly of APAF1 is dependent on the release of cytochrome *c* from the intermembrane space of mitochondria, a process that is dependent on the function of the two pro-apoptotic BAX-like proteins BAX and BAK. In healthy cells, the functions of BAX and BAK are inhibited by anti-apoptotic BCL2-like proteins such as BCL2, BCL-xL or MCL1. In response to apoptotic signals, pro-apoptotic BH3-only proteins such as BAD or BID are activated and promote cytochrome *c* release, apoptosome assembly and caspase activation by blocking anti-apoptotic BCL2-like proteins and possibly also directly activating BAX-like proteins (reviews) (45,227) (Figure 1). In nematodes and mammals, BH3-only proteins can therefore be regarded as key activators of apoptotic cell death (reviews) (113,147,217). As described in more detail below, BH3-only proteins indeed are often the targets of regulatory pathways that control programmed cell death during nematode and mammalian development.

As mentioned above, the activation of caspases in flies is similarly facilitated by an apoptosome. However, caspase activation in flies appears to be controlled through the availability of pro-caspases, rather than the assembly of the apoptosome. Specifically, in healthy cells, members of the IAP (IAP, inhibitor of apoptosis protein) family of proteins such as Diap1 and Diap2 cause the ubiquitylation of pro-caspases, thereby inactivating them. In response to apoptotic stimuli, RHG (RHG, Reaper, Hid, and Grim) proteins such as Reaper and Hid are activated, which leads to the Reaper- and Hid-dependent ubiquitylation and proteosomal degradation of IAP proteins (reviews) (83,110,194). Consequently, caspases such as Dronc and Drice, are activated in a Dark-dependent manner and initiate apoptosis (Figure 1). As outlined in more detail below, RHG and IAP proteins both represent key regulators of apoptotic cell death in flies and are targeted by pathways that control programmed cell death during fly development.

Autophagic cell death

At the morphological level, autophagic cell death is characterized by the presence of autophagic vesicles (autophagosomes) within the dying cells and the absence of engulfment through phagocytes, at least during early stages of the cell death process (reviews) (7,148). Compared to apoptotic cell death, little is known to date about the regulation and mechanisms of autophagic cell death. The best characterized example of this type of programmed cell death

is the death of the larval salivary gland during *D. melanogaster* metamorphosis (see below, 2.1). Genetic studies have revealed that the autophagic cell death of the salivary gland is dependent on genes not only involved in autophagy (or 'self-eating') but apoptosis (16,114, 131). Like apoptotic cell death in *D. melanogaster* (see above), the apoptotic 'branch' of the autophagic death of the larval salivary gland requires the activation of RHG proteins, which cause the ubiquitylation and proteosomal degradation of IAP proteins, thereby allowing caspase activation. Conversely, the autophagic 'branch' appears to be mediated by the cellular autophagy machinery. Autophagy has long been considered a survival process that can be activated in response to starvation (review)(107). Recently however, it has been recognized as a process that under certain circumstances can also contribute to the destruction of tissues such as the larval salivary gland of *D. melanogaster* (7,148). The cell biology and mechanism of autophagy has mainly been studied in the budding yeast *Saccharomyces cerevisiae* and in mammalian cells. Furthermore, genetic studies in *S. cerevisiae* led to the identification of about 30 *ATG* (*ATG*, autophagy-related genes) genes, the function of which in autophagy is conserved from *S. cerevisiae* to mammals (reviews) (144,201). The gene *ATG1*, which encodes a serine/threonine protein kinase, is a key regulator of autophagy induction. For example, the activation of its *D. melanogaster* homologue *Atg1* can be sufficient for the formation of autophagosomes, the fusion of autophagosomes with lysosomes and the subsequent degradation of autophagosomal content (7,148). The activity of the *Atg1* protein is negatively controlled by the serine/threonine protein kinase TOR (TOR, target of rapamycin) (review) (52). TOR activity in turn is regulated by signaling pathways such as the class I phosphatidylinositol 3-kinase (PI3K) signaling pathway. Specifically, in the presence of nutrients, PI3K-signaling results in the activation of TOR and *Atg1* repression, thereby blocking autophagy. Conversely, in the absence of nutrients, PI3K-signaling ceases and TOR is no longer activated, resulting in *Atg1* activation and autophagy induction. A number of *ATG* genes, including the *D. melanogaster ATG1* homologue *Atg1*, have been implicated in the autophagic death of the larval salivary gland of *D. melanogaster* (16,72,115,132). Furthermore, PI3K-signaling and TOR activity affect this autophagic cell death, suggesting that many of the players involved in the regulation and mechanism of autophagy in starving cells are also involved in autophagic cell death in dying cells (55) (see below, 2.1). However, what is currently unknown is how autophagy actually contributes to cell killing. One possibility is that the activation of lysosomal proteases acts in parallel to caspases to cause the complete dismantling of the doomed cells.

PROGRAMMED CELL DEATH DURING ANIMAL DEVELOPMENT

1. Deletion of cells formed in excess

The deletion of cells by programmed cell death is critical for the control of cell numbers and, hence, cellular homeostasis. This function of programmed cell death is particularly critical during animal development, since excess cells are generated during the development of a number of organ systems, such as the nervous system. The programmed death of neurons during the development of the sympathetic nervous system in mammals (1.1) and the programmed death of the NSM sister cells in *C. elegans* (1.2) are examples of how programmed cell death contributes to the generation of a functional nervous system. In the case of the development of the sympathetic nervous system in mammals, competition between neurons for pro-survival factors is a determining factor in the life-versus-death decision of individual neurons. In the case of the death of the NSM sister cells, the life-versus-death decision is determined by the asymmetric division of the NSM neuroblast, which gives rise to the NSM sister cell. Finally, the programmed death of epithelial cells in *D. melanogaster* (1.3) is an example of how programmed cell death is employed to regulate the sizes of organs and tissues. The Hippo signaling pathway turns out to control the size of epithelial tissues by concomitantly regulating cell death and cell proliferation.

1.1 Programmed cell death in the developing sympathetic nervous system in mammals—Programmed cell death is a critical aspect of the development of the vertebrate nervous system and it has been proposed that about 50% of all neurons initially generated are eliminated through this process (review)(154). This was recognized more than half a century ago by Hamburger and Levi-Montalcini, who proposed the neurotrophic hypothesis, which states that the survival of a particular neuron is dependent on its ability to successfully compete for limiting amounts of neurotrophic factors (79,121). The developing sympathetic nervous system, which is a branch of the peripheral nervous system, has since served as a model for studies on the mechanisms that control neuronal survival and death during mammalian development. These studies have elucidated some of the molecular mechanisms that promote survival or programmed cell death in developing sympathetic neurons (reviews)(19,70,77, 95). Using this model, molecular insight has also been obtained about the role that ‘competition’ plays in the life-versus-death decision in developing neurons (50).

In the developing sympathetic nervous system, target tissues, such as different types of muscles or glands, secrete neurotrophic factors and this results in their innervation by postganglionic sympathetic neurons. Neurons that successfully innervate the target tissue survive, whereas unsuccessful neurons die through BAX-, APAF1- and caspase-dependent apoptotic cell death (49,219). The extent of innervation depends on the amount of neurotrophic factor secreted by a specific target tissue, which indicates that the target tissue not only initiates but also controls this process (79,121). However, what are the mechanisms that orchestrate the complex interactions between the target tissue and an excess of developing neurons that all have the ability to innervate the target? The most prominent neurotrophic factor secreted by target tissues is nerve growth factor (NGF), which promotes neuronal survival (46,122). NGF binds to NTRK1 (TrkA), a member of the TRK receptor tyrosine kinase family, on the distal-most axon terminals of developing sympathetic neurons. The NGF-NTRK1 complex is subsequently internalized through an endocytic mechanism and transported to the cell body in the form of a ‘signaling endosome’. Once in the cell body, NGF induces a pro-survival transcriptional program (reviews) (85,246). (This long-distance communication between axon terminal and cell body is also referred to as ‘retrograde neurotrophic signaling’.) Specifically, NGF induces the transcriptional upregulation of the gene encoding NTRK1, which results in increased NTRK1 levels and presumably reinforced NTRK1-dependent pro-survival signaling (Figure 2) (50). As described in more detail below, this pro-survival mechanism most probably involves the activation of a RAS-, PI3K-, AKT-dependent pro-survival signaling pathway as well as the inactivation of a MLK-, JNK-dependent pro-apoptotic signaling pathway. However, simultaneously, NGF also appears to induce a pro-death transcriptional program. Specifically, NGF also induces the transcriptional upregulation of the genes encoding the neurotrophin receptor NGFR (p75NTR), which is a member of the tumor necrosis factor receptor (TNFR) superfamily, and the two neurotrophins brain-derived neurotrophic factor (BDNF) and neurotrophin 4 (NTF4 or NT-4) (Figure 2) (50). Interestingly, BDNF and NTF4 have been shown to promote the death of sympathetic neurons by binding to NGFR (11,50,224). (How NGFR induces cell death is also described in more detail below.) Therefore, NGF-induced changes in gene expression not only seem to strengthen NTRK1 pro-survival signaling (and, hence, the survival of a particular neuron), but make a particular neuron susceptible to autocrine or paracrine-induced BDNF-, NTF4-, NGFR-dependent death. Furthermore, they bestow a particular neuron with pro-death factors (BDNF and NTF4), which can induce cell death in neighboring neurons in a paracrine fashion. So, among the developing sympathetic neurons that all have the ability to innervate the target tissue, who wins and who loses? What appears to be critical is the strength of the NTRK1 signaling in a particular neuron, since NTRK1 signaling not only can promote survival but block NGFR-dependent cell death through a mechanism that has not been elucidated yet (130). Therefore, neurons that get a ‘head start’ on reinforcing NGF-dependent NTRK1 signaling might be protected from NGFR-dependent cell death and, for this reason, survive in the presence of BDNF and NTF4. Conversely, neurons

that fail to reinforce NTRK1 signaling before they synthesize NGFR might be susceptible and undergo programmed cell death in the presence of BDNF and NTF4 (Figure 2) (50).

Upon NGF withdrawal, developing sympathetic neurons undergo apoptotic cell death, which is dependent on BAX and the activation of caspases (49,51,146,165) and which can be blocked by BCL2 and BCL-xL (60,64,74). This observation suggests that NTRK1 signaling promotes survival at least in part by blocking an apoptotic program. It has now been established that NTRK1 signaling blocks apoptotic cell death by regulating the activities of at least two independent signaling pathways: (i) a pro-apoptotic pathway involving the kinases MLK (mixed lineage kinase) and JNK (c-JUN N-terminal kinase) and a member of the AP-1 family of basic/leucine zipper transcription factors, c-JUN; (ii) a pro-survival pathway involving the small GTPase RAS, the kinases PI3K and AKT and the FOXO transcription factor FOXO3a (review) (77). Both of these pathways target the transcriptional regulation of BH3-only genes, the products of which can trigger apoptotic cell death. (i) NTRK1 signaling blocks MLK-, JNK-dependent signaling. Upon NGF withdrawal, MLK, JNK signaling is activated, which results in JNK-dependent c-JUN phosphorylation and activation. Active c-JUN subsequently causes transcriptional activation of the *c-Jun* gene as well as the genes encoding the pro-apoptotic BH3-only proteins BIM and DP5 (17,18,81,167,207,212). Active JNK kinase also appears to phosphorylate BIM protein in response to NGF withdrawal thereby increasing its apoptotic activity (166). (ii) The *Bim* transcriptional control is also at least one target of the RAS, PI3K, AKT signaling pathway, which is activated by NTRK1 signaling. Upon NGF withdrawal, RAS, PI3K, AKT signaling is inactivated, which results in the dephosphorylation and activation of FOXO3a. Active FOXO3a subsequently causes the transcriptional activation of the *Bim* gene (Figure 2) (68).

The loss of *Bim* or *Dp5* function does not block NGF withdrawal induced apoptotic cell death as efficiently as the loss of *Bax* does (49,69,90,91,157,167,212). For this reason, BIM and DP5 might act redundantly to induce apoptotic cell death in response to NGF withdrawal. Alternatively, additional BH3-only proteins might be involved in this apoptotic cell death. Finally, the TRP53 (p53) tumor suppressor family members TRP63 (p63) and TRP73 (p73) have also been implicated in NGF withdrawal induced apoptotic cell death of sympathetic neurons (review) (95). Interestingly, while the loss of *Trp73* function enhances NGF withdrawal induced apoptotic death, the loss of *Trp63* function blocks NGF withdrawal induced apoptotic death (94,164). These observations suggest that TRP63 has pro- and TRP73 anti-apoptotic activity in developing sympathetic neurons. How TRP63 and TRP73 control NGF withdrawal induced apoptotic death remains to be elucidated however it has been proposed that TRP63 acts downstream of JNK to promote the transcriptional activation of the *Bax* gene.

The activation of NGFR by neurotrophins such as BDNF and NTF4 also results in the apoptotic death of sympathetic neurons (reviews) (13,48,176). Unlike NTRK1, which is a receptor tyrosine kinase, NGFR does not have catalytic activity. Instead, the interaction of neurotrophins with NGFR dimers appears to result in conformational rearrangements of the two disulfide-linked receptor subunits and the release of factors that are bound to their intracellular domains (210). More than 20 factors have been identified to date that interact with the intracellular domain of NGFR however the mechanism or mechanisms through which NGFR activation initiates apoptosis are still poorly understood. One of these interactors is the Krüppel-like zinc finger transcription factor NRIF (neurotrophin receptor interacting factor) (38,66,215). NRIF is critical for NGFR-induced apoptosis (38,124) and translocates into the nucleus upon NGFR activation (66). Furthermore, NRIF translocation appears to require the intracellular domain of NGFR, which is cleaved by γ -secretase in response to ligand-dependent NGFR activation (104). Finally, NRIF translocation is thought to also be promoted by the polyubiquitination of NRIF through the E3 ligase TRAF6, another interactor of the intracellular domain of NGFR

(Figure 2) (65). How NRIF activity promotes apoptosis in response to NGFR activation is currently unclear however it appears to involve the activation of JNK and TRP53 (p53). Specifically, JNK activation is critical for NGFR-induced apoptosis (37,80,226) and is abrogated in sympathetic neurons that lack *Nr1f* function (124). Furthermore, NGFR- or NRIF-induced apoptosis is also attenuated in sympathetic neurons that lack *Trp53* function (124). Since JNK and TRP53 both play a role in NGFR-induced apoptosis, this hints at the possibility that the JNK-responsive BH3-only genes *Bim* and *Dp5*, as well as the TRP53-responsive BH3-only genes *Puma* and *Noxa*, may play a role in this process; however this remains to be determined.

1.2 The programmed death of the NSM sister cells of *C. elegans*—During embryonic and post-embryonic development of a *C. elegans* hermaphrodite, 1090 somatic cells are generated of which 131 are reproducibly eliminated by programmed cell death (199,200). This phenomenon allows the genetic analysis of programmed cell death during *C. elegans* development at single cell resolution. The vast majority of those 131 cells differentiate into neurons, if prevented from dying, indicating that they are of neuronal origin. Hence, as in vertebrates, neuronal cells are initially formed in excess in *C. elegans* and their numbers are subsequently trimmed substantially by programmed cell death. One example of cells that are of neuronal origin and that are eliminated through EGL-1-, CED-4-, and CED-3-dependent apoptotic cell death during development are the two NSM (neurosecretory motoneuron) sister cells (42,57). About 410 min after the first cleavage of the *C. elegans* zygote, the two NSM neuroblasts each divide asymmetrically along the ventral/lateral to dorsal/medial axis of the embryo to give rise to a smaller cell located dorsal-laterally, the NSM sister cell, and a larger cell located ventral-medially, the NSM (82,200). (The two NSM neuroblasts are located on the left and right side of the animal's head region.) About 20 to 30 min after the completion of the NSM neuroblast division, the NSM sister cells have adopted morphological characteristics typical of apoptotic cells. In contrast, the NSMs survive and differentiate into serotonergic neurons. The apoptotic death of the NSM sister cells occurs in response to the transcriptional upregulation of the BH3-only gene *egl-1* gene, which is transcribed in the NSM sister cells, but not the NSMs (203). Both positive and negative regulators of *egl-1* transcription in the NSM lineage have been identified. A heterodimer composed of the daughterless-like and achaete-scute-like bHLH transcription factors HLH-2 and HLH-3 acts as a direct activator of *egl-1* transcription in the NSM lineage (203). Conversely, the Snail-related zinc finger transcription factor CES-1 can act as a direct repressor of *egl-1* transcription in the NSM lineage (58,139,203). HLH-2 and HLH-3 are detectable in both the NSMs and NSM sister cells; however CES-1 can only be detected in the larger NSMs (82,203). For this reason, it has been proposed that it is the absence of CES-1 protein from the NSM sister cell that triggers HLH-2-, HLH-3-dependent activation of *egl-1* transcription and, hence, its apoptotic cell death. In contrast, the presence of CES-1 protein in the NSM represses *egl-1* transcription, thereby allowing NSM survival.

Mutations that cause the NSM neuroblast to divide symmetrically rather than asymmetrically result in two daughter cells of similar sizes, both of which contain CES-1 protein and, consequently, survive (82). Therefore, the asymmetric presence of CES-1 in the NSM and NSM sister cell is determined by the asymmetric division of the NSM neuroblast. Three genes have so far been identified that most likely act in the NSM neuroblasts to cause their asymmetric division, *ces-2*, *dnj-11* and, surprisingly, also *ces-1* (58,82,138). *ces-2* and *dnj-11* encode a HLF-like bZIP transcription factor and a MIDA1-like chaperone, respectively. Both proteins act to reduce *ces-1* transcription in the NSM neuroblasts, thereby promoting asymmetric NSM neuroblast division (82). Interestingly, homologues of *dnj-11* and *ces-1* have previously been implicated in asymmetric cell division in other organisms. Specifically, the MIDA-1-like chaperone GlsA of the multi-cellular alga *Volvox carteri* is required for the visibly asymmetric cell divisions during embryogenesis that generate germ cells and somatic cells (141). In

addition, the CES-1-like Snail-related transcription factors Snail, Escargot and Worniu of *D. melanogaster* are involved in the asymmetric division of neuroblasts, which give rise to a neuroblast and ganglion mother cell (5,31). Snail, Escargot and Worniu affect asymmetric neuroblast division by promoting the expression of the genes *String* and *Inscuteable* (5). *String* encodes a CDC25-like protein phosphatase that is required for mitotic progression and *Inscuteable* encodes an adaptor protein that becomes localized in a polar fashion in the neuroblasts prior to their division and that is important for the displacement of the mitotic spindle along the cell division axis (reviews) (108,190). The polar localization of Inscuteable proteins is dependent on the conserved Par6/Par3/aPKC protein complex, which itself becomes localized in a polar fashion in response to epithelial polarity cues (reviews) (71,108). How *C. elegans* CES-1 affects the asymmetric division of the NSM neuroblast is currently unclear, but by analogy to the asymmetric division of *D. melanogaster* neuroblasts, one possibility is that it controls the expression of *C. elegans* homologues of *String* and *Inscuteable*. As is the case for *D. melanogaster* Snail, Escargot, and Worniu, the function of CES-1 is redundant with that of at least one unknown factor (58,82,139). However, the nature of this factor is still enigmatic. Furthermore, it is currently unclear whether the conserved Par6/Par3/aPKC protein complex, which was originally identified as required for the asymmetric division of the *C. elegans* zygote, plays a role in asymmetric NSM neuroblast division. Finally, how the NSM neuroblast becomes polarized is unclear, but cell non-autonomous signals are highly likely to play an important role.

Cells that are programmed to die during *C. elegans* development are thought to die in a cell-autonomous fashion i.e. without the involvement of non cell-autonomous, extra-cellular signals (review)(140). This notion is also correct for the NSM sister cell since, as a consequence of the absence of CES-1 protein in this cell, it is determined from the time the cell is generated that its fate is to undergo programmed cell death. However the decision to exclude CES-1 protein from the NSM sister cell is made in the NSM neuroblast and most likely involves extra-cellular signals that are required to polarize the NSM neuroblast along the ventral/lateral to dorsal/medial axis (82). Many of the 131 cells that are programmed to die during *C. elegans* development are thought to be the product of an asymmetric cell division (198). However, so far there are no indications that the *ces-2*, *dnj-11*, *ces-1* pathway plays a role in either the asymmetric divisions that give rise to these cells or their apoptotic death. It will therefore be interesting to determine the mechanisms and pathways involved in their life-versus-death decisions.

Finally, the role in apoptotic cell death of *ces-1* and *ces-2* appears to be conserved from nematodes to mammals. Specifically, it has been demonstrated that the mammalian homologues of CES-2 and CES-1, the proto-oncoprotein HLF (hepatic leukemia factor) and the Snail-related protein SNAI2 (SLUG), respectively, regulate the apoptotic death of pro-B cells in response to irradiation, which is dependent on the BH3-only gene *Puma* (92,93,221). Interestingly, HLF and SNAI2 have recently also been implicated in stem cell function (186). For this reason, it has been proposed that a pathway homologous to *ces-2*, *dnj-11*, *ces-1* pathway of *C. elegans* might function in mammals to control the asymmetric cell division of stem cells and the apoptotic fate of their daughters (82,147).

1.3 Control of tissue and organ size in *D. melanogaster* by programmed cell death—The control of cell numbers during development is also critical for the sizes of tissues and organs. Recently, a signaling pathway referred to as the Hippo (Hpo) pathway has been defined in *D. melanogaster* that controls the number of differentiating epithelial cells by coordinately regulating cell proliferation and caspase-dependent apoptotic cell death during development (reviews) (177,235). Hippo is a Ste20-type protein kinase that phosphorylates Warts (Wts), a nuclear DBF-2-related kinase. Hippo-mediated phosphorylation of Warts is facilitated by the WW-repeat containing protein Salvador (Sal), which might act as a scaffold

for Hippo and Warts. The activation of Warts' kinase activity is not only dependent on active Hippo kinase, but on 'Mob as tumour suppressor' (Mats), a kinase activator. Once activated, Warts phosphorylates the protein Yorkie (Yki), a transcriptional co-activator, thereby causing its inactivation. Since Yorkie lacks sequence specific DNA binding capabilities, it must act through one or more transcription factors. Recently, one such transcription factor was identified, the TEAD/TEF family transcription factor Scalloped (Sd), which mediates some of Yorkie's effects on cell proliferation and apoptotic cell death in at least some tissues (73,220, 233,237). Four transcriptional targets of Yorkie have so far been identified that are important for the control of cell proliferation and apoptotic cell death, *CycE*, *Bantam*, *Diap1*, and *Hid*. Yorkie promotes the transcription of *CycE*, which encodes a cyclin required for G1 to S phase transition, thereby promoting cell cycle progression (86). Furthermore, Yorkie promotes the transcription of the anti-apoptotic gene *Diap1* and represses the transcription of the pro-apoptotic RHG gene *Hid*, thereby repressing apoptotic cell death and promoting cell survival (86,151). Finally, Yorkie promotes the transcription of the miRNA gene *Bantam*, which has been shown to promote cell growth and cell cycle progression as well as cell survival (151, 205). While the targets of *Bantam* with respect to cell growth and proliferation remain to be identified, its target with respect to cell survival has been identified. *Bantam* directly targets *Hid* mRNAs thereby preventing their translation and apoptotic cell death (24). In summary, Hippo signaling restricts tissue and organ size by inhibiting Yorkie activity thereby promoting cell cycle arrest and apoptotic cell death. Conversely, the absence of Hippo signaling results in tissue and organ growth by activating Yorkie thereby promoting cell proliferation and growth and blocking apoptotic cell death.

How Hippo activity is regulated is not fully understood. Two plasma-membrane-associated, FERM (FERM, band 4.1/ezrin/radixin/moesin)domain-containing proteins, Merlin (Mer) and Expanded (Exp), are thought to act in parallel to promote the activity of the Hippo signaling pathway (78,160). Indeed, Expanded has recently been shown to directly interact with Yorkie and to sequester it in the cytoplasm, thereby causing its inactivation (6) (review) (236). The activities of Merlin and Expanded in turn most likely are under the control of the transmembrane protein and protocadherin Fat, which is likely to act as receptor for extracellular ligands or signals that regulate Hippo activity (15,40,189,209,213). Furthermore, the cadherin Dachshous (Ds)and the transmembrane kinase Four-jointed (Fj) have recently been implicated as ligands for Fat that transduce cell-to-cell signals to the Hippo pathway. Specifically, it has been proposed that Dachshous and Four-jointed detect differences in the levels of Dachshous or Four-jointed between neighboring cells and, hence, cell boundaries, which can lead to Hippo inactivation (214).

Finally, a signaling pathway homologous to the *D. melanogaster* Hippo pathway has been implicated in tumorigenesis in mammals, which suggests that the role of this pathway in the control of cell proliferation and apoptotic cell death might very well be conserved from *D. melanogaster* to mammals (177,235).

2. Deletion of cells 'no longer needed'

The deletion by programmed cell death of cells that are 'no longer needed' is a common feature of development and may be necessary for its proper progression. The removal by programmed cell death of the larval salivary gland during *D. melanogaster* metamorphosis is under the control of hormonal signaling and is probably the best understood example of this type of programmed cell death (2.1). While the regulatory mechanisms remain elusive, the recent analysis of the programmed death of the tail spike cell in *C. elegans* has resulted in some surprising findings about how programmed cell death can be initiated during *C. elegans* development (2.2).

2.1 Removal by programmed cell death of the larval salivary gland during *D. melanogaster* metamorphosis—

The larval salivary glands of *D. melanogaster* are removed through autophagic cell death and the action of genes involved in both autophagy and apoptosis are required for the process (97,114) (reviews) (7,148). More specifically, blocking either the ‘autophagic branch’ (through, for example, the inactivation of *Atg* genes) or ‘apoptotic branch’ (through, for example, blocking caspase function) partially blocks the destruction of the tissue, demonstrating that both processes are necessary for its complete removal (16,114,131). The programmed death of the salivary glands is triggered by a pulse of the steroid hormone 20-hydroxyecdysone (20E), which occurs about 12 hours after puparium formation (12 h APF) (APF, after puparium formation), and which also triggers pupation (Figure 3) (97,172). However, three additional events are critical to set the stage for efficient salivary gland death in response to the 20E pulse at 12 h APF. Those events are two earlier pulses of 20E at the mid-L3 (L3, 3rd instar larvae) transition (–24 h APF) and at puparium formation (0 h APF), and larval growth arrest as a result of the cessation of larval feeding at 0 h APF. The 20E pulses at –24 h and 0 h APF release brakes on the apoptotic branch, thereby rendering the salivary gland tissue competent for apoptosis in response to apoptotic stimuli. Growth arrest at 0 h APF releases a brake on the autophagic branch, thereby rendering the tissue competent for autophagy in response to appropriate signals.

The 20E pulse at 12 h APF, which triggers salivary gland death, results in the activation of the cellular receptor of 20E, EcR/USP, which is a heterodimer of two members of the nuclear hormone receptor family, EcR (NR1H1), and the RXR homolog, Ultraspiracle (USP, NR2B4) (109,204,223). Active EcR/USP promotes the transcriptional activation of the 20E-secondary-response genes *BR-C* (Broad Complex), *E74*, and *E93*, which encode a zinc finger-containing transcription factor, an ETS domain-containing transcription factor and a transcription factor with a novel DNA-binding motif, respectively (8,30,53). *BR-C*, *E74*, and *E93* subsequently cause the transcriptional activation of two important sets of genes: (i) pro-apoptotic genes, such as *Reaper*, *hid*, *Dark*, *Dronc* and *Drice* (32,72,98,105,115,116); (ii) genes involved in autophagy, such as *Atg-2*, *Atg-4*, *Atg-5*, *Atg-6*, *Atg-7*, *Atg-9* and *Atg-12* (Figure 3) (72,115). However, in order for the transcriptional upregulation of these sets of genes to lead to the initiation of autophagic cell death, the brakes on apoptosis and autophagy must first be released. Two brakes are on the apoptotic branch in larval salivary glands. Specifically, the tissue contains (i) a level of Diap1 protein that is sufficient to withstand the expression of both *Reaper* and *Hid* (225); (ii) active Fork head (Fkh) transcription factor, which actively represses the transcription of both *Reaper* and *Hid* (review) (206). Furthermore, the autophagic branch in feeding larvae is blocked by TOR activity (16,55). How are these brakes released?

The first 20E pulse at –24 h APF causes the activation of EcR as well as the transcriptional co-activator CPB (CREB binding protein), which together are required for the transcriptional repression of the *Diap1* gene. As a result, after –24 h APF, the level of Diap1 protein decreases to a level that no longer can withstand the expression in response to the third 20E pulse at 12 h APF of *Reaper* and *Hid* (Figure 3) (225). Furthermore, the transcriptional block on *Reaper* and *Hid* is removed in response to the second 20E pulse at 0 h APF. Specifically, the activation by 20E of EcR/USP leads to the transcriptional activation of *BR-C*, which subsequently causes the transcriptional repression of the *Fkh* gene (118,128), which encodes a potent transcriptional repressor of *Reaper* and *Hid* (36). As a result, reduced levels of Fkh protein allow the transcriptional activation of *Reaper* and *Hid* in response to the activation of *BR-C*, *E74* and *E93* in response to the third 20E pulse at 12 h APF (Figure 3) (36). In summary, at the time of the third 20E pulse at 12 h APF, the transcriptional upregulation of *Reaper* and *Hid* results in the inactivation of Diap function and the Dark-mediated activation of the caspases *Dronc* and *Drice*. How about the autophagic branch? The brake on autophagy is lifted after larvae cease to feed, which causes a general growth arrest at around 0 h APF. This growth arrest leads to the activation of the Hippo signaling pathway (see above, 1.3) (55). The activation of this

pathway blocks PI3K signaling, which results in TOR inactivation. The inactivation of TOR most probably leads to the activation of Atg1, which has been shown to be sufficient for the induction of autophagy in the larval salivary gland, thereby removing the brake on autophagy potentially by causing the (16). In response to the third 20E pulse at 12 h APF, the transcriptional activation of *Atg* genes subsequently leads to the activation of autophagy. Finally, the activation in response to the third 20E pulse at 12 h APF of both caspases as well as autophagy causes the removal by autophagic cell death of the salivary gland (Figure 3).

Autophagic cell death is a common phenomenon during amphibian and insect metamorphosis when entire organs or tissues are removed (41,181). However, autophagic cell death has also been observed during vertebrate development. For example, the involution of mammary and prostate glands or the regression of the Müllerian ducts during male development are likely to occur through autophagic cell death as well (41,181). Whether the process of autophagic cell death in *D. melanogaster* and vertebrates is conserved at the molecular level remains to be determined.

2.2 The programmed death of the tail spike cell of *C. elegans*—Most cells that are programmed to die during *C. elegans* development die as undifferentiated cells 20–30 min after being generated and have no known function during their short life time (199,200). Furthermore, most cells that are programmed to die do so in response to the transcriptional upregulation of the BH3-only gene *egl-1*, which results in the activation of the caspase CED-3 (43,57,127,163,203). Finally, most cells that are programmed to die do not contain a transcriptionally active *ced-3* gene and therefore presumably inherit the proCED-3 protein from their mothers (135). (Caspases are synthesized as inactive pro-enzymes and are matured to the fully active caspase through the action of APAF1-like adaptor proteins.) However, the tail spike cell, a binucleated cell that is generated through the fusion of two hypodermal cells, ABplppppppa and ABprppppppa, is an exception. The tail spike cell survives for 300 min and differentiates before it undergoes apoptotic cell death (200). Furthermore, the tail spike cell appears to have a function before it is eliminated. By forming the ‘tail spike’, a narrow spike of cuticle that extends to the very tip of the tail, the tail spike cell may play an important role in the morphogenesis of the worm’s tail. The tail spike cell also represents an exception with respect to the mechanism that triggers its apoptotic death. Whereas the loss of the BH3-only gene *egl-1* almost completely blocks the death of most cells that are programmed to die during development, it only blocks the death of about 30% of the tail spike cells (135). Furthermore, unlike in most cells that are programmed to die, the *ced-3* gene is transcriptionally active and proCED-3 protein presumably made in the tail spike cell prior to its death (135). The notion that *ced-3* transcriptional upregulation is necessary to trigger the death of the tail spike cell is furthermore supported by the finding that mutations that specifically reduce *ced-3* transcriptional upregulation in the tail spike cell can block its death. More specifically, loss-of-function mutations in the gene *pal-1*, which encodes a Caudal-like homeodomain transcription factor, reduce *ced-3* caspase transcription in the tail-spike cell and block the death of about 50% of the tail spike cells. Furthermore, the PAL-1 protein most probably acts as a direct activator of *ced-3* transcription in the tail spike cell (135).

The *pal-1* gene is best known for its role in cell fate specification and patterning in posterior regions of the embryo (56,87). Specifically, through the control of *pal-1* mRNA translational, the PAL-1 transcription factor becomes targeted to the somatic descendants of the posterior blastomere P2, where it activates a transcriptional network that specifies the lineages generated by the two blastomeres C and D (14,87). In that context, PAL-1 activity is modulated by WNT/MAP kinase signaling. Specifically, depending on the nuclear level of the WNT/MAP kinase pathway effector POP-1 (the *C. elegans* homologue of TCF), PAL-1 either promotes the hypodermal or body wall muscle fate (14,61). The tail spike cell is not a descendant of either the C or D blastomere (it is a descendant of the AB blastomere), but it is located in the most

posterior region of the embryo where, as mentioned above, it most likely plays a role in tail morphogenesis (200). For this reason, it will be interesting to determine how *pal-1* function is regulated in the tail spike cell to control *ced-3* transcriptional activation and, hence, the death of the tail spike cell. PAL-1 protein can be detected in the tail spike cell several hours before the tail spike cell dies (135). For this reason, PAL-1 might not be sufficient to activate *ced-3* transcription in the tail spike cell or might require activation at the post-translational level just prior to its death.

Why would the transcriptional upregulation of the caspase gene *ced-3* rather than the BH3-only gene *egl-1* be critical for the initiation of apoptotic cell death in the tail spike cell? While we don't have an answer yet, I propose the following model. Most cells that are programmed to die during *C. elegans* embryogenesis do so 20–30 min after they are generated (200). Since these cells inherit proCED-3 protein from their mothers, the level of proCED-3 inherited from the mother must be sufficient for apoptotic death, if a cell dies within 20–30 min (135). However, the tail spike cell survives for 300 min instead of 20–30 min after it is generated (200). Therefore, as a result of protein turn over, the level of proCED-3 inherited from the mother may constantly decrease in the tail spike cell until it reaches a level that no longer is sufficient for apoptotic cell death. In order to increase the level of proCED-3 again, the *ced-3* gene might therefore be transcriptionally activated in the tail spike cell prior to its death. What is unclear at this point is whether *egl-1* is also transcriptionally upregulated in the tail spike cell prior to its death. The observation that *egl-1* is not completely dispensable for the death of the tail spike cell suggests that *egl-1* transcriptional upregulation might still be a requirement for apoptosis initiation at least in some tail spike cells (30%). In the remaining tail spike cells, *ced-3* might be upregulated to a level that is sufficient for *egl-1*-independent cell death. (The overexpression of *ced-3* has been shown to be sufficient for the death of cells that normally survive (184).) Interestingly, the transcriptional activation of the caspase genes *Drice* and *Dronc* of *D. melanogaster* is also critical for certain cell death events during development, which indicates that the mechanism through which the apoptotic death of the *C. elegans* tail spike cell is initiated is not an exception (32,33,105).

The surprising story of the tail spike cell emphasizes the fact that we know very little about the mechanisms that insure that core components of the apoptosis machinery required for apoptotic cell death (APAF1-like adaptors, caspase) are ubiquitously present. These components are believed to be present in most if not all cells of multicellular animals, but how this is achieved is currently unclear. The notion that it is crucial to have these components present at sufficient levels is demonstrated by the studies regarding the tail spike cell death.

3. Deletion of cells to sculpt the organisms

The deletion by programmed cell death of cells for the purpose of sculpting the organism is also a common theme of development. A classic example of this type of programmed cell death is interdigital cell death during the development of vertebrate limbs (3.1). Growth factor signaling, which is important for patterning the vertebrate limb, turns out to play an important role in this process. The generation of sexual dimorphism in the nervous system through programmed cell death is another example of how programmed cell death is used to sculpt an organ. Recently, molecular insight has been obtained about how programmed cell death is used to sculpt the *C. elegans* nervous system in hermaphrodites and males (3.2). Not too surprisingly, the *C. elegans* sex determination pathway and its most downstream effector are essential for this process.

3.1 Interdigital cell death during the development of the vertebrate limb—The removal by programmed cell death of fetal interdigital mesenchyme during the development of vertebrate limbs that lack webbing is a classic example of how programmed cell death is

used to sculpt developing organisms (10,88,230). Interdigital cell death is accompanied by hallmarks of apoptotic cell death such as DNA fragmentation, cytochrome *c* release and caspase activation and, hence, occurs through apoptotic cell death (96,230,242).

Digits form in the distal or 'autopodial' region of the limb bud from undifferentiated mesenchymal cells that are found just underneath a layer of ectodermal cells that form the apical rim of the limb bud and that is referred to as the apical ectodermal ridge (AER). These undifferentiated mesenchymal cells have chondrogenic potential and either undergo chondrogenic differentiation, thereby contributing to the formation of digital rays and, hence, digits, or form part of the interdigital mesenchyme, which is subsequently removed by apoptotic cell death (reviews)(150,208). Members of the superfamily of transforming growth factor β (TGF β) –like growth factors (TGF β , activin, bone morphogenetic proteins [BMPs]) play critical signaling roles at different stages during vertebrate limb development (review) (171). With respect to apoptotic cell death, BMPs play a particularly important role. BMPs are required for the apoptotic death of undifferentiated mesenchymal cells in the interdigital regions referred to as 'interdigital necrotic zones' (INZs). More specifically, several BMPs such as BMP2, BMP4, BMP5 and BMP7 are expressed in the interdigital regions and their overexpression in these regions promotes apoptotic cell death (63,240,245). Conversely, their inactivation through the expression of BMP inhibitors such as Noggin or Gremlin (76,137, 142,162) or through targeted gene inactivation in mice (12,103,106,182) causes a defect in interdigital cell death, thereby resulting in the inappropriate persistence of interdigital 'webs', which is also referred to as 'soft tissue syndactyly'. Furthermore, BMP2, BMP4, BMP5 and BMP7 most likely signal through the BMP receptor Ia (BMPRI), a serine/threonine receptor kinase, since its inactivation can also block interdigital cell death (175). How the activation of BMPRI leads to apoptosis induction is not fully understood. However, there is evidence that BMPRI activation leads to the phosphorylation of the proteins SMAD1, SMAD 5, and SMAD8 and their subsequent assembly with the protein SMAD4, which then translocates into the nucleus to activate gene transcription (Figure 4) (review) (243).

However, BMP signaling is also critical for chondrogenic differentiation of undifferentiated mesenchymal cells that will form the digital rays (reviews) (102,171). For this reason, BMP signaling cannot be sufficient to specify the apoptotic fate in the interdigital mesenchyme. Indeed, other signaling pathways have been implicated in interdigital cell death. For example, fibroblast growth factor (FGF) signaling, which is required for limb outgrowth (review) (133), is also involved in this process. Specifically, the administration of FGF can block interdigital cell death, which suggests that FGF signaling might antagonize BMP-dependent interdigital cell death (28,63,129,149). However, it has also been shown that the administration of FGF can promote interdigital cell death and that the inhibition of FGF signaling through the use of inhibitors can block interdigital cell death, which suggests that FGF signaling might cooperate with BMP signaling to cause interdigital cell death (Figure 4) (62,142). One possible explanation for these contradicting results is that the effect of FGF signaling on interdigital cell death might change during limb developmental. Recently, genetic studies in the mouse shed some light on the mechanism through which FGF signaling can antagonize BMP-dependent interdigital cell death. It was demonstrated that the inactivation specifically in the AER of the *Bmpr1* gene results in the upregulation in the AER of FGF4 and FGF8 and a block in the apoptotic death of the interdigital mesenchyme (155). This finding suggests that BMP signaling might not only induce apoptotic cell death in a cell-autonomous manner in the interdigital mesenchyme, but in a cell non-autonomous manner by affecting FGF signaling in the AER, which then in turn blocks apoptotic cell death in the interdigital mesenchyme (Figure 4). FGF signaling in the AER, and FGF8 expression in particular, might be controlled by Notch signaling, which has been shown to affect interdigital cell death in a cell non-autonomous manner (59,99,156). Furthermore, retinoic acid (RA) signaling plays a role in interdigital cell death as well. Mice that are homozygous for a null mutation in the gene encoding the retinoic

acid receptor γ (RAR γ) and either homozygous or heterozygous for a null mutation in the gene encoding RAR β , exhibit soft tissue syndactyly (54,67). Furthermore, the exposure of interdigital mesenchyme to RA can induce premature apoptosis (173). The mechanism through which RA signaling affects interdigital cell death is currently unclear however RA has been shown to promote the expression in the interdigital mesenchyme of BMP7 (Figure 4) (54, 173).

How do all these signaling pathways induce apoptotic cell death in the interdigital mesenchyme? No apoptotic pathway has so far been delineated but the analysis of knock-out mice has revealed some candidate players. Mice lacking both *Bax* and *Bak* function exhibit soft tissue syndactyly, demonstrating that they are required for interdigital cell death (123). The anti-apoptotic *Bcl2* gene is expressed at low levels in interdigital regions but at high levels in digital rays, suggesting that the BCL2 protein might antagonize the activities of BAX and BAK in digits thereby preventing their apoptotic death (152). Furthermore, mice lacking *Bax* and the BH3-only gene *Bim* also have webbed paws, indicating that the BIM protein might play a critical role in the initiation of apoptotic cell death in the interdigital mesenchyme (Figure 4) (89). Interestingly however, in mice lacking *Apaf1* or *caspase* function, interdigital cell death is only delayed and eventually occurs through caspase-independent, necrotic cell death rather than apoptotic cell death (39). This observation suggests that in the absence of active caspases, BAX and BAK induce a necrotic cell death pathway, which can also lead to the programmed destruction of the interdigital mesenchyme. This necrotic pathway might involve the activation of lysosomal proteases such as cathepsins (Figure 4) (244). What remains to be determined is how BMP, FGF, NOTCH, RA and possibly additional signaling pathways act in concert to regulate the activity of, for example, BIM. In addition, since mice lacking *Bim* function do not exhibit soft tissue syndactyly, additional BH3-only proteins are likely to be involved in this process. Finally, it will be interesting to determine the mechanism through which BAX and BAK induce the programmed destruction of the interdigital mesenchyme in the absence of caspase activity.

3.2 The generation of sexual dimorphism in the *C. elegans* nervous system through programmed cell death—Two sexes exist in *C. elegans*, males and self-fertilizing hermaphrodites (25). (*C. elegans* hermaphrodites are basically females that can produce sperm for a limited period of time late during larval development.) Males and hermaphrodites exhibit extensive sexual dimorphism (reviews) (218,231). For example, the nervous system of hermaphrodites contains 302 neurons, eight of which are found specifically in hermaphrodites. In contrast, males contain 381 neurons, 87 of which are found specifically in males. Most of the dimorphism observed is a result of differences in cell fate or the number of cell divisions that occur within a certain cell lineage. However, two hermaphrodite-specific neurons and four male-specific neurons are sex-specific as a result of EGL-1-, CED-4-, and CED-3-dependent apoptotic cell death during embryonic development. Specifically, the two hermaphrodite-specific neurons (HSNs) are hermaphrodite-specific as a result of the apoptotic death of the HSNs in males. Conversely, the four cephalic companion neurons (CEMs) are male-specific as a result of the apoptotic death of the CEMs in hermaphrodites (200). Sex in *C. elegans* is determined by the ratio of the number of X chromosomes to the number of pairs of autosomes (X:A ratio) (218,231). This primary signal determines the level of transcription of the gene *her-1*, which encodes a secreted protein. In animals with only one X chromosome (X0), *her-1* is transcribed and HER-1 protein is generated, which binds to its transmembrane receptor TRA-2, thereby inactivating it. The inactivation of TRA-2 results in the assembly of an E3 ubiquitin ligase complex of the CBC (CUL-2, Elongin B, and Elongin C) type (composed of the proteins FEM-1, FEM-2, FEM-3, ELC-1, CUL-2 and RBX-1), which causes the proteasomal degradation of the GLI-like zinc finger transcription factor and global determinator of sexual fate, TRA-1 (193,232) (review) (112). TRA-1 promotes female development by repressing the transcription of genes required for male development (218,

231). Therefore, in XO animals, TRA-1 activity is low or absent, resulting in the development of a male. Conversely, in animals with two X chromosomes (XX), the *her-1* gene is not transcribed (or transcribed at a low level) and the amount of HER-1 protein generated is not sufficient to inactivate TRA-2. As a consequence, TRA-2 prevents the assembly of the E3 ubiquitin ligase complex and, hence, the proteasomal degradation of TRA-1. Additionally, in XX animals, TRA-1 protein is processed through a currently unknown mechanism to generate a degradation-resistant, hermaphrodite-specific isoform, which acts as a transcriptional repressor capable of repressing male-specific genes (179). Therefore, XX animals develop as females.

By controlling the transcription of the pro-apoptotic BH3-only gene *egl-1*, TRA-1 specifies sexually dimorphic cell death events. Specifically, TRA-1 directly represses *egl-1* transcription in the HSNs, thereby causing HSN survival in hermaphrodites, but not males (43) (Figure 5). Conversely, TRA-1 directly represses the transcription of the gene *ceh-30*, which encodes a Bar homeodomain transcription factor that promotes CEM survival by repressing (directly or indirectly) *egl-1* transcription, thereby causing CEM survival in males, but not hermaphrodites (158,180) (Figure 5). Active TRA-1 is ubiquitously present throughout development and adult life in hermaphrodites however it only affects the *egl-1*-dependent, apoptotic death of the HSNs and CEMs. Therefore, it remains to be determined how TRA-1's ability to control the transcription of *egl-1* and *ceh-30* and, hence, its apoptotic function is restricted to these two types of neurons. It has been speculated that TRA-1 might antagonize HSN- and CEM-specific transcriptional activators of *egl-1* and *ceh-30*, however such factors have so far remained elusive. One possible explanation for why these factors are still elusive is that they might be required for the normal specification and/or differentiation of these neurons, which would make it difficult to identify them using standard genetic approaches.

4. Deletion of potentially harmful cells

The deletion by programmed cell death of potentially harmful cells is critical throughout development and adult life. One important example of this type of programmed cell death is the elimination during T cell development of autoreactive thymocytes by a process referred to as 'negative selection' (4.1). The identification and specific elimination of autoreactive thymocytes has recently been shown to be dependent on the ability of these thymocytes to interact with medullary thymic epithelial cells. The elimination of cells damaged by DNA damage-inducing agents is another important function of programmed cell death that is required for the maintenance of genome integrity and cellular homeostasis. Interestingly, the ability of *D. melanogaster* to respond to DNA damage by inducing programmed cell death is developmentally regulated (4.2). This regulation appears to be mediated by epigenetic changes in enhancers that mediate the transcriptional activation of pro-apoptotic genes in response to DNA damage.

4.1 Negative selection of autoreactive thymocytes in mice—About 95% of all thymocytes (T cell precursors) generated are eliminated by programmed cell death through processes referred to as 'positive' and 'negative' selection. During positive selection, thymocytes are identified that exhibit on their cell surface T cell receptor complexes (TCR) that can interact with major histocompatibility complexes (MHC) on the cell surfaces of dendritic cells or cells of the thymic stroma. Thymocytes that fail these criteria are eliminated by programmed cell death. During negative selection or 'clonal deletion', thymocytes are identified that can recognize self-antigens bound to MHCs. The elimination of such 'autoreactive thymocytes' is critical for normal immune system function and the prevention of autoimmunity and occurs through APAF1- and caspase-dependent apoptotic cell death (185,202). How are these autoreactive thymocytes recognized? Negative selection takes place predominantly in the medulla of the thymus and medullary thymic epithelial cells (mTECs)

play a particularly important role in the process. mTECs exhibit on their cell surface MHC complexes with many different tissue- and organ-specific antigens that are normally not found in thymocytes or mature T cells, but are present in other tissues and organs, such as the nervous system or the pancreas. The gene AIRE (autoimmune regulator), which was originally identified as defective in human patients suffering from multiple organ autoimmunity autoimmune polyendocrinopathy (APECED) and which encodes a protein with structural motifs that implicates it in transcriptional regulation and chromatin modifications, is required for the ability of mTECs to express these tissue- and organ-specific antigens (reviews) (134, 161) (44,143). Specifically, mice lacking a functional AIRE gene, fail to express these antigens in mTECs, which results in the inappropriate survival of autoreactive T cells that subsequently attack various tissues and organs (2,3,126). This observation demonstrates that negative selection is important for the prevention of autoimmunity.

The activation or 'stimulation' of TCRs through their interaction with self-antigens bound to MHCs on mTECs triggers events in the thymocytes that lead to their apoptotic cell death (reviews) (192,197). The regulatory pathways involved in triggering the apoptotic death of thymocytes in response to TCR stimulation have not yet been completely delineated, however some of the critical players have been identified. TCR stimulation leads to a flux of calcium into the thymocyte, which, through a currently unknown mechanism, results in the transcriptional upregulation of the BH3-only gene *Bim* (9,23,35,145,178). Mice lacking *Bim* function exhibit a defect in negative selection of thymocytes, which demonstrates that *Bim* is required for the apoptotic death of autoreactive thymocytes (21,211). TCR stimulation also results in the post-translational modification of the BIM protein, probably phosphorylation (22,29). This post-translational modification might be caused by JNK kinase, which is activated after TCR stimulation in a manner that is possibly dependent on the Ste20-related kinase MINK (Misshapen-Nck-interacting kinase-related kinase) (119,136,166,170). An increased level of BIM protein and/or modified BIM protein subsequently results in the activation of BAX and BAK, which, like BIM, are required for negative selection (169), thereby leading to apoptosome formation and caspase activation. It is highly likely that BIM's pro-apoptotic function is mediated through its binding to anti-apoptotic members of the BCL2 family such as BCL-xL, which is the anti-apoptotic BCL2-like protein that is most abundantly expressed in thymocytes, or BCL2. However, the role of anti-apoptotic BCL2-like proteins in negative selection remains to be fully clarified since the overexpression of either BCL-xL or BCL2 in thymocytes only partially blocks negative selection (75,183,188,195,196,216). It has recently been demonstrated that the overexpression of BCL2 in T cells results in a concomitant increase in the level of BIM protein (101). One possible explanation for the observation that BCL2 overexpression fails to efficiently block negative selection therefore is that it simultaneously leads to an increase in BIM protein, thereby allowing the cell to remain sensitive to apoptotic stimuli.

The flux of calcium after TCR stimulation also causes the transcriptional upregulation of the gene *Nur77* (111,125,241). *Nur77* and the *Nur77*-related genes *Nurr1* and *Nop1* form a family of transcription factors that have also been implicated in the negative selection of thymocytes. Specifically, a dominant-negative NUR77 mutant protein, which blocks the activity of all three family members, blocks negative selection of autoreactive thymocytes when expressed in T lymphoid cells (34,239). How this family of transcription factors promotes the apoptotic death of autoreactive thymocytes is currently unclear however they most likely act in parallel to BIM rather than upstream.

4.2 DNA damage-induce programmed cell death during *D. melanogaster*

embryogenesis—The removal by programmed cell death of potentially harmful cells is important for cellular homeostasis. For example, cells that have acquired damaged DNA as a result of DNA damage-inducing reagents such as irradiation, either arrest in the cell cycle or

undergo apoptotic cell death (reviews) (174,238). Interestingly, at least in the fruit fly *Drosophila melanogaster*, the ability to induce apoptotic cell death in response to irradiation is controlled in a developmental stage-specific manner. For example, while embryos induce apoptotic cell death in response to irradiation until 7 hours after egg laying (AEL) (corresponding to developmental stages 1–11), they lose this ability 7–9 hours AEL and no longer induce apoptotic cell death in response to irradiation after 9 hours AEL (developmental stage 12 and higher) (4,222). Irradiation-induced apoptotic cell death in embryos 7 hours AEL and younger is dependent on the *D. melanogaster* orthologue of the tumor suppressor TRP53 (p53), DmP53 (26,100,153). DmP53 is required for the transcriptional upregulation in response to irradiation of the two pro-apoptotic RHG genes *Reaper* and *Hid*, the gene products of which induce apoptotic cell death in a caspase-dependent manner (26,27,117,191). The transcriptional upregulation of *Reaper* and *Hid* in response to irradiation is dependent on a ~30 kb *cis*-acting element (referred to as ‘irradiation responsive enhancer region’ or IRER), which is located immediately upstream of the *Reaper* transcription unit and ~240 kb upstream of the *Hid* transcription unit (234). (The pro-apoptotic genes *Hid*, *Grim*, *Reaper* and *Sickle* of *D. melanogaster* are all located within a ~270 kb genomic region (reviews) (83,110,194).) The IRER also includes a putative TRP53 response element (26). Interestingly, in embryos older than 9 hours AEL, DmP53 no longer can mediate the transcriptional upregulation of *Reaper* and *Hid* in response to irradiation. This lack of transcriptional response is specific to *Reaper* and *Hid* since the DmP53-dependent transcriptional upregulation in response to irradiation of the genes *ku70* and *ku80* is maintained after 9 hours AEL (234). The gene-specific loss of transcriptional response appears to be caused by epigenetic modifications. Specifically, starting at around 7 hours AEL, the IRER upstream of the *Reaper* transcription unit is subject to H3K9 and H3K27 trimethylation, which causes its open chromatin confirmation to adopt a heterochromatin-like structure (234). The *Reaper* and *Hid* transcription units maintain an open confirmation thereby maintaining their transcriptional responsiveness to stimuli other than irradiation. The inactivation of factors required for H3K9 and H3K27 trimethylation, i.e. the histone methyltransferase Su(var)3–9 and components of the Polycomb repressive Complex 2 (PRC2), respectively, causes a delay in the acquisition of resistance to irradiation-induced apoptotic cell death, indicating that these epigenetic marks are important for the acquisition of resistance (234). At a later stage of development (during pupae formation), sensitivity to irradiation is regained (4) and *reaper* and *hid* transcription are upregulated again in response to irradiation (27). Whether this phenomenon is a result of the reversal of the epigenetic changes introduced at the IRER 7–9 hours AEL remains to be determined. Finally, what triggers the acquisition of resistance to irradiation in embryos 7–9 hours AEL in the first place? Early embryogenesis is a time of rapid proliferation characterized by major mitotic waves. In contrast, later stages of embryogenesis are characterized by cellular differentiation. For this reason, the acquisition of a heterochromatin-like structure at the IRER occurs at a time when many cells lose their pluripotency and become restricted in their cell fate most probably as a result of epigenetic changes. The changes at the chromatin level that occur at the IRER therefore might occur as part of a major wave of chromatin remodeling that occurs during this critical stage of *D. melanogaster* development.

SUMMARY LIST

1. The control of programmed cell death during animal development is complex. While we have acquired a comprehensive understanding of the mechanisms of apoptotic cell death and are beginning to elucidate the mechanisms of autophagic cell death, we are only starting to realize how complex the regulation of programmed cell death during animal development is. Just to give an example, the BH3-only gene *egl-1* of *C. elegans*, which is required for the death of many of the 131 cells that undergo programmed cell death during *C. elegans* development,

most probably is the transcriptional target of at least 60–70 disparate pathways or combinations of pathways that control *egl-1* transcription in a cell-specific manner (147). To date, only a handful of these pathways have been identified molecularly (43,82,127,158,163,180,203). This observation suggests that the transcriptional regulation of mammalian BH3-only genes such as *Bim*, *Puma* or *Noxa*, will at least be equally complex.

2. Programmed cell death during animal development is controlled by pathways that have additional important functions during development. The *D. melanogaster* Hippo pathway, for example, not only controls the programmed death of epithelial cells but their ability to proliferate (177,235). Furthermore, NGF-dependent NTRK1-signaling is involved in determining the life-versus-death decision of developing sympathetic neurons, but also promotes the extension and branching of axons (70). And finally, BMP-signaling is not only required for the programmed death of interdigital mesenchyme but the chondrogenic differentiation of mesenchyme that will subsequently form digital rays (102,171). This phenomenon makes the analysis of the cell death function of these regulatory pathways challenging. However, it also demonstrates that programmed cell death is an integral and essential aspect of animal development.
3. At least some of the pathways involved in controlling programmed cell death during animal development are conserved. For example, a pathway homologous to the pathway that determines the death of the NSM sister cells in *C. elegans* functions in mammals to control the programmed death of pro B-cells (147). In addition, it is highly likely that the mammalian Hippo pathway not only regulates cell proliferation but also programmed cell death (235). I predict that future studies will reveal that many of the regulatory pathways that are currently being unraveled will turn out to be conserved among nematodes, flies and mammals.
4. Much more is still too learn about programmed cell death during animal development. New experimental models will help us discover new functions of programmed cell death, increase our understanding of its regulation, and provide insight into how this process evolved through animal evolution. For example, studies on freshwater polyps (*Hydra*) and planarians have already revealed that programmed cell death plays an important role in stem cell function and regeneration (20,159). In addition, with the zebrafish *Danio rerio*, a vertebrate model of programmed cell death has become available that is amenable to forward genetic analysis (1,168). Indeed, analyses in *D. rerio* have already provided new insight into, for example, the regulation of DNA damage-induced apoptotic cell death (187). Finally, the analysis of programmed cell death in *Hydra*, a member of the phylum Cnidaria, which includes some of the simplest multi-cellular animals, will allow us to determine how programmed cell death during animal development arose. It will also allow us to get a glimpse of how this process evolved during animal evolution to constitute the programmed cell death pathways that we know today and that have been delineated predominantly through studies performed in *C. elegans*, *D. melanogaster* and mouse.

Glossary

Mini-glossary

Apoptotic cell death	Caspase-dependent cell death; also referred to as programmed cell death type I
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Autophagic cell death	A cell death mechanism that depends on the activities of both genes involved in autophagy and genes involved in apoptosis; also referred to as programmed cell death type II
Autophagy	A process through which cells can digest intracellular organelles and content ('selfeating')
Necrotic cell death	A cell death mechanism that is independent of caspases
List of important acronyms used	

BH3	BCL2 homology domain 2
IAP	Inhibitor of apoptosis protein
RHG	Reaper, Hid, Grim

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Figure 1. Apoptotic pathways in *C. elegans*, *D. melanogaster*, and mammals

Simplified schematics are shown. Key regulators of the pathways are shown in orange. See text for details (INTRODUCTION).

Figure 2. Programmed cell death in the developing sympathetic nervous system in mammals
 A simplified model is shown. Molecular events in two individual developing neurons (not shaded and shaded) are depicted early and late in the life-versus-death decision. The BH3-only genes *Bim* and *Dp5* are indicated in orange. NTRK1 (TrkA), NGFR (p75NTR), NTF4 (NT-4), TRP53 (p53), TRP63 (p63), TRP73 (p73). See text for details (PROGRAMMED CELL DEATH DURING ANIMAL DEVELOPMENT, 1.1).

Figure 3. Removal by programmed cell death of the larval salivary gland during *D. melanogaster* metamorphosis

A simplified model is shown. Molecular events in response to the 20E pulses at -24 h, 0 h and 12 h APF are depicted. The IAP gene *Diap1* and the RHG genes *Reaper* and *Hid* are indicated in orange. Genes required for autophagy are indicated in blue. Below the schematic is a summary of the presumptive protein levels of *Reaper*, *Hid*, *Diap1*, *Atg1* and other *Atg* proteins. Proteins involved in apoptosis are depicted in orange and proteins involved in autophagy in blue. See text for details (PROGRAMMED CELL DEATH DURING ANIMAL DEVELOPMENT, 2.1).

Figure 4. Interdigital cell death during the development of the vertebrate limb

A simplified model is shown. Molecular events in the apical ectodermal ridge (AER) (shaded) and interdigital mesenchyme (not shaded) are shown. The BH3-only protein BIM is depicted in orange. Dashed lines indicate interactions that have not yet been demonstrated experimentally and/or the mechanisms of which have not yet been elucidated. The green lines depict the pro-apoptotic function of FGF signaling in interdigital cell death and the purple lines its anti-apoptotic function. Notch and RA signaling affect interdigital cell death by controlling the levels of FGF8 in the AER and of BMP7 in the interdigital mesenchyme, respectively. See text for details (PROGRAMMED CELL DEATH DURING ANIMAL DEVELOPMENT, 3.1).

Figure 5. The generation of sexual dimorphism in the *C. elegans* nervous system through programmed cell death

A simplified model is shown. Molecular events in males and hermaphrodites are depicted. The BH3-only gene *egl-1* is shown in orange. See text for details (PROGRAMMED CELL DEATH DURING ANIMAL DEVELOPMENT, 3.2).