Apoptotic and Nonapoptotic Caspase Functions in Animal Development

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A developing animal is exposed to both intrinsic and extrinsic stresses. One stress response is caspase activation. Caspase activation not only controls apoptosis but also proliferation, differentiation, cell shape, and cell migration. Caspase activation drives development by executing cell death or nonapoptotic functions in a cell-autonomous manner, and by secreting signaling molecules or generating mechanical forces, in a noncell autonomous manner.

Programmed cell death or apoptosis occurs widely during development. During *C. elegans* development, 131 cells die by caspase CED-3-dependent apoptosis; however, *ced-3* mutants do not show significant developmental defects (Ellis and Horvitz 1986). In contrast, studies on caspase mutants in mouse and *Drosophila* have revealed caspases' roles in development. During development, cells are exposed to extrinsic and intrinsic stresses, and caspases are activated as one of multiple stress responses that ensure developmental robustness (Fig. 1). Caspases actively regulate animal development through both apoptosis and nonapoptotic functions that involve cell–cell communication in developing cell communities (Miura 2011). This chapter focuses on the *in vivo* roles of caspases in development and regeneration.

INITIATOR CASPASES

Caspase-8

Caspase-8 is a death-effector domain (DED)-containing initiator caspase, initially found as a component of the Fas/Apo-1 death-inducing signaling complex (DISC) (Muzio et al. 1996) and a MORT1/FADD-binding protein (Boldin et al. 1996). Caspase-8-knockout mice are lethal at embryonic day (E)11.5 (Varfolomeev et al. 1998; Sakamaki et al. 2002; Kang et al. 2004). At E10.5, abnormal yolk sac vasculature is observed, followed by various defects in the developing heart and neural tube (Varfolomeev et al. 1998; Sakamaki et al. 2002). Mutant embryos show hyperemia in the superficial capillaries and other blood vessels, and extensive erythrocytosis in the liver. These defects result in severe

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Figure 1. Caspase activation during development. An embryo undergoes intrinsic and extrinsic stress, which activates caspases to execute both apoptotic and nonapoptotic functions, including cell differentiation and dendrite pruning. Apoptotic cells affect the shape and behavior of their neighboring cells. Caspase-activated cells are shown in dark gray.

primary or secondary depletion of the hematopoietic precursor pool. Mutant embryos also show impaired heart muscle development (Varfolomeev et al. 1998).

The homozygous mutant neural and heart defects are rescued by ex vivo whole-embryo culture during E10.5–E11.5 (Sakamaki et al. 2002), suggesting that the mutant phenotypes of the neural tube and heart are caused by secondary effects of impaired angiogenesis in the yolk sac. Consistent with this idea, endothelium-specific *caspase-8*-knockout mice show the same phenotype as conventional knockout mice (Kang et al. 2004). Whereas knockout mice of Fas, TNFR1, DR3, or TRAIL are viable (Pfeffer et al. 1993; Rothe et al. 1993; Adachi et al. 1995; Liu et al. 2001; Wang et al. 2001; Cretney et al. 2002), FADD- and cFLIPL-deficient mice show the same phenotypes as *caspase-8*-knockout mice (Yeh et al. 1998, 2000; Zhang et al. 1998). Thus, the *caspase-8* developmental phenotype does not seem to depend on any single death ligand or receptor.

Recent studies revealed that *caspase-8*/FADD pathway has an important role in suppressing RIPK1/RIPK3-mediated necrosis (necroptosis). Deletion of RIPK1 or RIPK3 rescued lethality of FADD or *caspase-8* deletion (Kaiser et al. 2011; Oberst et al. 2011; Zhang et al. 2011).

The inactivation of RIPK1 or RIPK3 through *caspase-8*-mediated cleavage may be required for the protection of cells from necroptosis, thus *caspase-8* deletion can activate necroptosis pathway during development (Feng et al. 2007; Cho et al. 2009).

Caspase inhibitors (pan caspase-inhibitor Z-VAD, baculovirus p35, or cowpox virus CrmA) block macrophage differentiation and activation (Sordet et al. 2002) in vitro. This finding and studies in myelomonocytic-lineage-specific *caspase-8*-knockout mice suggest that the M-CSF-stimulated differentiation of macrophage precursors is *caspase-8*-dependent. In the absence of *caspase-8*, such precursors are eliminated by apoptosis, indicating that *caspase-8* has a prosurvival role (Kang et al. 2004). Upon M-CSF stimulation, *caspase-8* mediates cleavage of serine/threonine kinase receptor-interacting protein 1 (RIPK1), which prevents the sustained activation of NF κ B and leads to macrophagic differentiation (Rebe et al. 2007).

Signal exchanges among epidermal keratinocytes, dermal fibroblasts, and leukocytic cells are critical regulatory mechanisms for skin homeostasis and wound healing. These reciprocal interactions are initiated by the paracrine signaling of interleukin 1 α (IL1 α), which activates both skin stem-cell proliferation and

cutaneous inflammation. During wound healing, caspase-8 expression is downregulated in the epidermal granular layer, after which a p38-MAPK-mediated upregulation of NLRP3 leads to inflammasome assembly and caspase-1 activation, resulting in the noncanonical secretion of IL1 α from epidermal keratinocytes (Lee et al. 2009b). Mature IL1 α stimulates NF κ B, which leads to growth arrest and promotes keratinocyte survival. IL1 α also stimulates dermal fibroblasts to secrete secondary factors such as KGF and GM-CSF, which signal back to the epidermis to induce keratinocyte proliferation in the basal layer. Caspase-8's importance is supported by the observation that *caspase-8*-deficient skin shows epidermal hyperplasia (Lee et al. 2009b). The chronic loss of epidermal *caspase-8* in mice enhances wound-healing responses and recapitulates atopic dermatitis (Li et al. 2010a). Consistent with this, *caspase-8* is increased in diabetic mice in which wound healing is impaired (Al-Mashat et al. 2006).

Caspase-9 and Dronc

Caspase-9 and *apaf-1* are key components of the intrinsic, mitochondrial apoptosis pathway. On a mixed 129/SvJ background, the knockout of *apaf-1* or *caspase-9* causes similar developmental defects, including neural-tube closure (NTC) defects in the hindbrain and enlarged ventricular zones in the fore- and mid-brain (Cecconi et al. 1998; Hakem et al. 1998; Kuida et al. 1998; Yoshida et al. 1998). Live imaging of caspase activation in cultured mouse embryo revealed the detailed apoptotic process of NTC. Two types of caspase-activated cells appeared during NTC. One is a typical apoptotic cell (classical [C]-type), and the other is an atypical apoptotic cell (dancing [D]-type), which does not show cell fragmentation and remains and dances around their original sites for a long period. Caspase-activation kinetics in D-type is slower than in C-type and caspase-7 is selectively activated in D-type. *Apaf-1*-deficiency abolished the appearance of both C- and D-type cells, and delayed the progression of neural tube closure. Thus inhibition of apoptosis could cause the NTC defect and in-

crease the risk of exencephaly (Yamaguchi et al. 2011).

Semaphorin 7A (Sema7A) that can promote axon guidance and neuronal migration in neural development is a direct substrate for caspase-9 (Ohsawa et al. 2009). *Caspase-9* and *apaf-1* mutant mice show misrouted axons, impaired synaptic formation, and defects in olfactory sensory neuron (OSN) maturation without changes in the OSN cell number (Ohsawa et al. 2010). Thus, some developmental abnormalities resulting from caspase inhibition could be attributable to the loss of a nonapoptotic function of caspase-9.

Dronc is a *Drosophila* ortholog of caspase-9/CED-3, and the only CARD-containing caspase in *Drosophila*. The *dronc* mutation abolishes most of the programmed cell death during development and causes pupal death (Chew et al. 2004; Daish et al. 2004; Waldburger et al. 2005; Xu et al. 2005; Kondo et al. 2006). Dronc is essential for diap1-degradation-induced cell death (Leulier et al. 2006), compensatory proliferation, and dendrite pruning, described below.

EXECUTIONER CASPASES

Caspase-3, Caspase-7, Drice, and dcp-1

Caspase-3 deficiency in the 129/SvJ mouse causes neurodevelopmental abnormalities, including an expanded ventricular zone, ectopic neural structures, and gross brain malformations, as observed in *caspase-9*- or *apaf-1*-knockout mice (Kuida et al. 1996; Leonard et al. 2002). Caspase-3 and caspase-7 are both categorized as executioner caspases, although their substrate specificity is somewhat different. Caspase-7 is a more selective protease than caspase-3 (Walsh et al. 2008). The developmental defect of executioner caspase, *apaf-1* and *caspase-9*, is strain-dependent in mice. C57BL/6-dominant background mice with a single knockout of *caspase-3* or *caspase-7* do not show severe developmental defects and have a normal life span; however, mice with double knockouts of *caspase-3* and *caspase-7* (DKO) die shortly after birth, indicating that these caspases have redundant roles in embryonic development (Leonard

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et al. 2002; Lakhani et al. 2006). A small percentage (~10%) of them showed exencephaly, but the investigators assumed that this was attributable to residual genes from 129/SvJ background, as the DKO mice were backcrossed only six generations onto C57BL/6 background, which was probably insufficient to remove all of the residual genes from 129/SvJ background (Lakhani et al. 2006). Most of the DKO mice did not show any sign of exencephaly and their cause of death after birth was unclear but might be caused by defective heart development. Caspase-7's *in vivo* function has not been extensively studied, but evidence suggests it regulates inflammation and pathological cell death (Teixeira et al. 2008). Caspase-7, but not caspase-3, is a direct substrate for caspase-1 (Lamkanfi et al. 2008), and caspase-7 is involved in the induction of *Legionella pneumophila*-infected macrophages (Akhter et al. 2009).

Drice is a caspase-3 ortholog in *Drosophila*, in which it is the most abundantly expressed effector caspase (Fraser and Evan 1997; Kumar and Doumanis 2000). *Drice* mutants show pupal lethality and significantly decreased apoptosis during development, but the reduction is not as severe as in *dronc* mutants (Kondo et al. 2006; Muro et al. 2006; Xu et al. 2006). This is because *drice* shares functional redundancy with the other executioner caspase, *dcp-1*. *dcp-1* mutants are viable and fertile (Kondo et al. 2006; Muro et al. 2006; Xu et al. 2006). *drice/dcp-1* double mutants phenocopy *dronc* mutants, indicating that these two executioner caspases are functionally redundant, downstream of *dronc* (Kondo et al. 2006).

During metamorphosis, caspases are induced and activated by ecdysone, and most larval tissues including the salivary gland, midgut, and larval epidermis, are removed (Baehrecke 2000; Takemoto et al. 2007). *p35* expression can only partially suppress the salivary-gland cell death; the inhibition of autophagy is required to block tissue degeneration completely (Berry and Baehrecke 2007). Despite high caspase activity in the midgut, autophagy but not caspase activity is essential for the programmed midgut cell death (Denton et al. 2009).

Caspase-Mediated Apoptosis in Fine-Tuning Neural Cell Populations

Large numbers of cells are thought to be eliminated by apoptosis in neural development (Buss et al. 2006). Live imaging enables a death-fated cell to be monitored throughout its lifetime. During sensory organ development of the *Drosophila* notum, about 20% of the differentiating neural cells die (Koto et al. 2011). Detailed observations of these cells indicate that the excess neural progenitors do not die in a random manner; instead, which proneural gene *neuralized*-positive cells die follows a rule and depends on the Notch-activation level. Thus, Notch-regulated apoptosis of aberrant, mis-specified sensory organ precursor (SOP) mediates the fine-tuning of SOP cell selection (Koto et al. 2011). It has been proposed that apoptosis-mediated cell selection eliminates harmful autoreactive lymphocytes in primary lymphoid tissues (Strasser et al. 2008). Thus, apoptosis-mediated error-correction systems may be critical for eliminating aberrant cells, achieving normal development, and maintaining tissue homeostasis.

Caspases in Morphogenesis

One of the most fundamental roles of cell death during development is in tissue sculpting. However, none of the mouse mutants of core regulators of apoptosis, except for *bax/bak* double-mutant mice or *bid/bim/puma* triple mutant mice, show severe defects in morphogenetic cell death, such as the classic example of interdigital tissue removal (Lindsten et al. 2000; Ren et al. 2010). Furthermore, although cultured cells from *apaf-1*, *bax/bak*, or *bid/bim/puma* mutant mice have significant defects in cell-stress-induced apoptosis, *apaf-1*, *bax/bak*, or *bid/bim/puma* mutant mice generally develop into mostly normal adults, depending on their genetic background (Chautan et al. 1999; Lindsten and Thompson 2006; Ren et al. 2010). These results indicate that caspase-mediated apoptosis is dispensable, or that an alternative cell-death mechanism removes cells when the apoptotic machinery is inhibited. In fact, nonapoptotic cell death is observed in the interdigital

region and other areas in *apaf-1* mutant mice (Chautan et al. 1999; Cande et al. 2002; Nagasaka et al. 2010). However, the caspase functions in mammalian morphogenetic processes remain to be studied in detail.

Genetic studies of caspase signaling in *Drosophila* revealed some interesting roles of caspases in morphogenetic processes. For example, apoptotic cell death may help generate the mechanical forces that drive cell movements and cell-shape changes during epithelial morphogenesis. During *Drosophila* embryonic development, a process called dorsal closure completes the sealing of the yolk and the amnioserosa. As the dorsal epidermis spreads and amnioserosa cells constrict, multiple forces drive the closure (Hutson et al. 2003). Apoptosis of amnioserosa cells may help generate one of the forces needed to seal the dorsal epithelium over the embryo (Toyama et al. 2008).

When a discontinuity in Dpp activity is generated between neighboring cells, JNK-dependent apoptosis is triggered to restore the appropriate cell-positional information (Adachi-Yamada et al. 1999). Similar mechanism is involved in the morphogenesis of the *Drosophila* leg joint (Manjon et al. 2007). During normal segmentation of the *Drosophila* distal legs, Dpp is expressed in the most distal part of every forming segment, and only diffuses proximally; the sharp boundary in Dpp activity induces JNK-dependent apoptosis, which leads to the epithelial folding that prefigures the adult joint (Manjon et al. 2007).

During *Drosophila* embryonic development, the Hox protein Deformed helps maintain the boundary between the maxillary and mandibular head lobes, by activating the cell-death-promoting gene *reaper* to induce localized apoptosis. Abdominal-B also regulates segment boundaries through the regional activation of apoptosis. Thus, apoptosis induced by *Drosophila* Hox genes modulates segmental morphology (Lohmann et al. 2002).

Global tissue rotation is a morphogenetic movement involved in controlling tissue elongation (Haigo and Bilder 2011). *Drosophila* apoptosis mutants show an orientation defect of the male terminalia. The male terminalia nor-

mally rotates 360° during development, and incomplete rotation causes the orientation defect of male terminalia (Macias et al. 2004). During development, the male genitalia rotation accelerates to complete the full 360° rotation; this acceleration is impaired in apoptosis-defective flies (Abbott and Lengyel 1991; Benitez et al. 2010; Suzanne et al. 2010; Kuranaga et al. 2011). Genetic and live-imaging studies indicated the active roles of apoptosis in genitalia rotation (Suzanne et al. 2010; Kuranaga et al. 2011) and caspase drives the acceleration of genitalia rotation to complete the morphogenesis of male genitalia within a limited developmental window (Kuranaga et al. 2011).

Caspases in Compensatory Proliferation and Regeneration

Some animal tissues, including the limbs of urodele amphibians such as newts and salamanders, the tadpole tail of *Xenopus*, the heart and fin of fish (Johnson and Weston 1995; Akimenko et al. 2003; Poss 2007), *Drosophila* larval imaginal discs, and freshwater hydra, have a striking regenerative capacity. When such tissues are injured, many cells die by apoptosis. This apoptosis appears to be essential for the subsequent regenerative cell proliferation in hydra (Chera et al. 2009), planarians (Hwang et al. 2004; Pellettieri et al. 2010), *Drosophila* (Milan et al. 1997; Huh et al. 2004a; Ryoo et al. 2004), *Xenopus* (Tseng et al. 2007), newt (Vlaskalin et al. 2004), and mouse (Li et al. 2010c). Upon healing, a tissue's final shape and size often match those of the uninjured tissue, owing to a regenerative cell proliferation mechanism that is often called "compensatory proliferation" (Fig. 2).

The local induction of apoptosis by ectopic toxin expression in *Drosophila* wing imaginal disc results in elevated cell proliferation around the site of apoptosis, suggesting that cells can perceive apoptosis in their vicinity and undergo extra cell divisions until the original cell number is restored (Milan et al. 1997). When the *Drosophila* cell-death-inducing gene *reaper* (*rpr*) or *head involution defective* (*hid*) is expressed, or imaginal discs are subjected to X rays together with baculovirus *p35* (a potent inhibitor of

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Figure 2. Cell competition (CC) and compensatory proliferation (CP). In CC, a cell that receives less growth factor is eliminated by apoptosis. Dying cells release growth factors that promote the CP of neighboring cells.

effector caspases such as drice and dcp-1 but not of the initiator caspase dronc [Meier et al. 2000; Xu et al. 2009]), the apoptosis-signaling cascade is activated only to the point in which dronc has been activated, and the cells fail to undergo apoptosis. Under these conditions, the proliferation

regulators Wingless (Wg) and Decapentaplegic (Dpp) are ectopically expressed (Huh et al. 2004a; Perez-Garijo et al. 2004, 2005; Ryoo et al. 2004; Kondo et al. 2006; Wells et al. 2006), and hyperplastic tissue growth occurs. In this experimental system, *dronc* was found to be required for proliferation and was suggested to be involved in the induction of mitogen expression in the proliferating cells of wing imaginal discs (Huh et al. 2004a; Kondo et al. 2006; Wells et al. 2006). In the differentiating cells of eye imaginal discs, effector caspases trigger the activation of Hedgehog (Hh) signaling for the compensatory proliferation (Fig. 3) (Fan and Bergmann 2008).

Classical tissue regeneration experiments were performed using *Drosophila* imaginal discs (Hadorn and Buck 1962; Hadorn et al. 1968). These tissue-ablation experiments required microsurgery skills and were technically difficult. Recently, nonsurgical, genetic ablation methods were used to study imaginal disc regeneration (Smith-Bolton et al. 2009; Bergantinos et al. 2010). Apoptosis induced in a portion of the wing imaginal disc by the transient expression of the cell-death-inducing gene *reaper* (diap1 antagonist) or *eiger* (TNF α ortholog) caused caspase activation and the extrusion of dying

Figure 3. Genetic cascade of compensatory proliferation in proliferating or differentiating cells in *Drosophila* imaginal discs (wing and eye discs).

cells from the epithelium. As the tissue healed, cytoskeletal reorganization was observed with JNK activation at the leading edges of the healing tissue, not in the dying cells; furthermore, JNK activation was required for compensatory proliferation of the regenerating tissue (Bergantinos et al. 2010). Unlike the proliferation observed when *reaper* and caspase inhibitor *p35* are coexpressed in the wing imaginal disc, *Wg* is predominantly expressed in the proliferating cells, not in the dying cells during tissue regeneration (Smith-Bolton et al. 2009).

The relationship between apoptosis and compensatory proliferation was studied in *Hydra* during the regeneration after amputation (Chera et al. 2009). Mid-gastric bisection induces apoptosis specifically in the head, and not in the foot-regenerating region. Apoptotic cells in the head-regenerating region release *Wnt3* to trigger compensatory proliferation and regeneration. The ectopic induction of apoptosis by heating in the foot-regenerating region activates the *Wnt3*/ β -Catenin pathway, and is sufficient to cause head formation. In both cases, treatment with the pan-caspase inhibitor Z-VAD abolishes the *Wnt3*/ β -Catenin pathway activation and head regeneration. These results showed that caspase-mediated signaling promotes compensatory proliferation in *Hydra* tissue regeneration.

In a mammalian wound-healing model, *caspase-3*- or *caspase-7*-deficient mice show defects in skin-wound healing and cell proliferation (Li et al. 2010c). *Caspase-3* and *caspase-7* were also important for healing in partial-hepatectomy model mice. In *caspase-3*- or *caspase-7*-knock-out mice, the regenerative cell proliferation was reduced about 50%, indicating that executioner caspases are required for cell proliferation during wound healing and tissue regeneration (Li et al. 2010c). The growth signal from the caspase-activated cells might be prostaglandin E2 (PGE2). Calcium-independent phospholipase A2 (iPLA2), which produces arachidonic acid and triggers PGE2 production, is activated after cleavage by caspase-3 (Atsumi et al. 1998; Zhao et al. 2006). PGE2 is known to promote stem-cell proliferation (Feher and Gidali 1974; Liou et al. 2007; North et al. 2007), tissue regenera-

tion (Goessling et al. 2009), and wound healing (Kuhner et al. 1986; Paralkar et al. 2003).

Caspase in Tissue Growth Control

During regeneration, the overall size of the recovering tissue is regulated. The mechanism of tissue-growth control has been studied in *Drosophila* wing discs. The central regulator of tissue size is insulin/TOR signaling, which ultimately controls the ribosome and protein synthesis. Conditional expression of the ribosome-inactivating protein Ricin toxin A chain in the posterior wing imaginal disc causes a nonautonomous reduction in the growth and proliferation rates of adjacent cell populations (Mesquita et al. 2010). In this condition, *dronc* activation, detected by an antibody against activated human caspase-3 (Fan and Bergmann 2009), is observed not only in the Ricin-expressing territories but also in adjacent cells (Mesquita et al. 2010). The coexpression of baculovirus *p35* in the Ricin-expressing domain prevents cell death in that region and partially prevents it in the neighboring region. Thus, the cell proliferation is nonautonomously rescued, suggesting that effector caspases in the growth-depleted territory are required for the reduced cell proliferation in the adjacent cells. However, the nonautonomous reduction of overall tissue size is not rescued by the expression of *p35*.

p53 is activated in the growth-deficient cells, and effector caspases are activated downstream of *p53*. As with *p35* expression, a reduction in *p53* activity in the Ricin-expressing territory nonautonomously rescues cell proliferation. However, unlike *p35* expression, the adjacent tissue growth is rescued by *p53* suppression. Targeted Ricin-expression activates *p53*, which induces caspase activation. The tissue size and cell proliferation are regulated by *p53* nonautonomously, but caspases have a unique role in regulating the proliferation of adjacent cell populations (Mesquita et al. 2010).

Caspase in Cell Competition

Cell competition was first discovered by genetic studies in *Drosophila* (Morata and Ripoll 1975;

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Simpson 1979; Simpson and Morata 1981). The genes for ribosomal proteins (RPs) are essential, and their homozygous mutations are lethal; however, their heterozygotes can result in the “Minute” dominant, haploinsufficient phenotypes, such as a slow growth rate and short and thin bristles (these heterozygous flies are called “Minute” because of their small bristles). When mosaic clones consisting of wild-type cells and *Minute* cells are generated in wing imaginal discs, the *Minute* cells are selectively eliminated by apoptosis, and wild-type cells eventually take over within a compartment (Fig. 2) (Morata and Ripoll 1975; Moreno et al. 2002). Thus, there is a competitive interaction between *Minute* cells (losers) and wild-type cells (winners). This elimination of *Minute* cells does not occur under starvation conditions (Simpson 1979), suggesting a link between the *Minute* mutation and nutrition control. Target of rapamycin complex 2 (TORC2) regulates cell survival and growth. TORC2 is activated by directly associating with a ribosome, suggesting there might be a direct link between *Minute* and growth-control mechanisms (Zinzalla et al. 2011). Cells carrying different dosages of the *myc* gene also show competitive interaction (de la Cova et al. 2004; Moreno and Basler 2004). High-*myc* cells are winners and low-*myc* cells are losers under competitive conditions. Because *myc* promotes the gene expression of ribosomal proteins, super-competition by high *myc* might result from high ribosomal biosynthesis (Johnston 2009).

There are differences in the cell-competition processes induced by *Minute* and *myc*. The induction of cell death in *Minute* clones is initiated locally, whereas *myc*-induced competition affects up to 10 cells (de la Cova et al. 2004; Li and Baker 2007). Both *Minute* and *myc*-induced competition preserves the wing size, indicating a balance between the growth of winner and the loss of loser cells. However, cell competition itself is not a major regulatory mechanism for wing size control. Rather, growth arrest that occurred once compartment size reached the final dimension plays significant role for size control (Martin et al. 2009).

The concept of cell competition can be extended to explain the competitive nature of cell

populations in the body (Baker 2011). Mosaic clones of tumor-suppressor genes that encode proteins essential for establishing epithelial apicobasal polarity, such as *scribble* (*scrib*), *discs large* (*dlg*), and *lethal giant larvae* (*lgl*), are eliminated by cell competition (Woods and Bryant 1991; Agrawal et al. 1995; Brumby and Richardson 2003; Igaki et al. 2006). The engulfment of loser cells by winner cells is required for cell death execution and cell elimination in both the *Minute* and *scribble* models of cell competition (Li and Baker 2007; Ohsawa et al. 2011). JNK is important for elimination of *scribble* clone, but caspases may not be (Brumby and Richardson 2003; Igaki et al. 2006), suggesting that caspase-dependent and -independent mechanisms are used for cell elimination in different types of cell competition. Caspase-independent elimination of *scribble* clones by cell competition is executed by Eiger/TNF signaling (Igaki et al. 2006, 2009). Cell death signaling by Eiger/TNF has a similarity with necroptosis (Degterev et al. 2005; Christofferson and Yuan 2010) and is regulated by JNK and energy metabolic pathway (Kanda et al. 2011).

TISSUE REMODELING DURING DEVELOPMENT

Although cell competition has been extensively studied by mosaic analysis in *Drosophila* wing imaginal discs, studies on the physiological nature of cell competition are still limited. One system for studying cell-competition-like phenomena in vivo is the abdominal epithelial replacement in *Drosophila*. The adult abdominal epidermis is formed by histoblasts, which replace the polyploid larval epidermal cells (LECs) during the pupal stage. About 15 h after puparium formation, the histoblast nests expand, and the histoblasts migrate collectively over the abdomen (Madhavan and Madhavan 1980). As in cell competition, the histoblasts behave like winners and replace the LECs, which undergo apoptosis, like losers. During abdominal epithelial replacement, the coordination of LEC apoptosis and histoblast proliferation enables the orderly substitution of cell types without changing the area of the epithelium, as in

cell-competition experiments in wing discs. Possible mechanisms for the replacement of abdominal epithelium include mutual signaling events, mechanical forces, and cell competition (Ninov et al. 2007, 2010). A study of caspase activation during epithelial replacement in vivo at single-cell resolution indicated that caspase activation in LECs is induced at the LEC/histoblast boundary, which expands as the LECs die. Transition from the S/G2 phases is necessary to induce nonautonomous LEC apoptosis at the LEC/histoblast boundary. This replacement boundary, formed as caspase activation is regulated locally by cell–cell communication, may drive the dynamic orchestration of cell replacement during tissue remodeling by competitive interaction (Fig. 4) (Nakajima et al. 2011).

Nonapoptotic Caspase Functions

Caspases exert nonapoptotic functions, which include roles in cell proliferation, migration, differentiation, and immunity (Kuranaga and Miura 2007; Yi and Yuan 2009). These functions are executed via three mechanisms.

Local Activation of Caspases

During neural development, some axons and dendrites degenerate or are pruned to create proper neural circuits. In *Drosophila*, dendritic

pruning in class IV dendritic arborization sensory neurons occurs during metamorphosis. During this process, caspase activity is detected locally in the degenerating dendrites, and mutation of the initiator caspase *dronc* preserves most of the dendrite morphology (Kuo et al. 2006; Williams et al. 2006; Schoenmann et al. 2010). Mutations of effector caspases, such as *drice* or *dcp-1*, or expression of caspase inhibitor *p35*, only weakly reduce dendritic pruning, suggesting that *dronc* promotes dendritic pruning by a mechanism other than the activation of effector caspases (Schoenmann et al. 2010). The *dronc* activation in this system is regulated by a *diap1*-degrading kinase, IK2/DmIKKe (Kuranaga et al. 2006; Lee et al. 2009a). Neuromuscular degeneration caused by disruption of the spectrin/ankyrin skeleton requires *dronc*, *dcp-1*, *apaf-1* (*dark*), and *bcl-2* family gene *debcl* (Keller et al. 2011). In contrast, caspase inhibition by *p35* or *diap1* fails to block axon pruning during *Drosophila* metamorphosis (Awasaki et al. 2006).

Caspases are reported to be involved in axon degeneration in mammals. Caspase-6 but not caspase-3 is activated after NGF deprivation in dissociated dorsal root ganglion (DRG) culture (Nikolaev et al. 2009), and caspase-6 inhibition alone can block the axonal degeneration. In a different DRG culture system, NGF deprivation induces axonal degradation and the activation

Figure 4. Ordered caspase activation during epidermal remodeling in *Drosophila* metamorphosis. Caspase-activated cells (dark grey and arrow) at the “replacement boundary” are eliminated, and histoblast (small cell) cell-cycle progression is coupled with larval epidermal cell (large cell) apoptosis. Apoptosis at the replacement boundary creates a spatiotemporal pattern of tissue removal, and maintains the epithelial integrity as histoblasts replace larval epidermal cells.

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of both caspase-3 and caspase-6 in axons (Schoenmann et al. 2010). To completely protect the axons from degenerating, NAD^+ must be added to the DRG culture with caspase-6 or caspase-3 inhibitors. Because an NAD^+ -sensitive pathway is thought to be involved in Wallerian degeneration (Araki et al. 2004), caspase-mediated and NAD^+ -sensitive pathways may act separately or in concert to control axonal degeneration. BAX acts upstream of both NAD^+ and caspase pathways in DRG culture, and is required to eliminate misprojecting sensory axons in Plexin-A3/A4 double-knockout mice (Schoenmann et al. 2010).

In *Xenopus*, caspase-3 is required for the growth cones in cultured retinal cells to respond to the chemotropic molecules Netrin-1- or lysophosphatidic acid (LPA) (Campbell and Holt 2003).

During sperm differentiation in *Drosophila*, 64 haploid spermatids of each cyst are connected by cytoplasmic bridges. To form individual sperm, these bridges are removed, and most of the cytoplasm is expelled. This process is called “individualization.” Caspases are activated in the individualization complex, and their spatially dependent activation is necessary to complete this process (Arama et al. 2003, 2006, 2007; Huh et al. 2004b; Kaplan et al. 2010).

Regulation of Endogenous Caspase Inhibitors

Three *Drosophila* genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, are key regulators of apoptosis during *Drosophila* embryogenesis (White et al. 1994). The overexpression of one of the three genes induces apoptosis via a caspase-activation pathway. Each of these proteins binds to diap1, an endogenous caspase inhibitor, through interactions mediated by their NH_2 terminus. Genetic and biochemical data indicate that one way these proteins promote apoptosis is by inhibiting diap1’s ability to prevent caspase activity (Gyrd-Hansen and Meier 2010; Sandu et al. 2010). The mammalian mitochondrial proteins Smac/DIABLO and HtrA2/Omi, whose truncated NH_2 terminus shares similarity with Rpr, Hid, and Grim, similarly inhibit the anti-apoptotic function of XIAP

and enhance caspase activation (Gyrd-Hansen and Meier 2010).

A genetic modifier screen for genes that regulate caspase activation identified *Drosophila* IKK-related kinase (DmIKK ϵ /IK2) as a diap1-degrading kinase. DmIKK ϵ is a homolog of the noncanonical I κ B kinases (IKK ϵ /IKKi or NAK/T2K/TBK1), which regulate NF- κ B activation or interferon regulatory factor (IRF)-3 and -7 activation in mammals (Kawai and Akira 2006). A low-level caspase activity is detected in the *scabrous*-expressing proneural clusters in wild-type wing discs (Kanuka et al. 2005). Similar to the phenotype of *p35* or *dronc-DN* overexpression in proneural clusters, *DmIKK ϵ* knockdown leads to the generation of one extra bristle in the scutellar region (Kuranaga et al. 2006). Thus, DmIKK ϵ appears to control the level of diap1, which determines the nonapoptotic caspase activity required for defining the number of bristles as two in the proneural cluster of the scutellar region (Kuranaga et al. 2006). In addition, diap2 controls the basal caspase-activity level for both apoptotic and nonapoptotic functions (Ribeiro et al. 2007).

Temporal Caspase Activation

A transient increase in caspase-3 activity is observed during neural-cell differentiation from neurospheres or PC12 cells (Rohn et al. 2004; Fernando et al. 2005). Caspase-3 and caspase-8 are transiently activated after the transduction of iPSC-inducing transcription factors, and caspase-3 or caspase-8 inhibition in human fibroblast cells prevents the iPSC induction, suggesting that transient, nonapoptotic caspase activities have critical roles in cell-fate reprogramming (Li et al. 2010b).

The diap1 expression is a critical regulator for the temporal caspase activation (Koto et al. 2009). To investigate the spatial and temporal pattern of diap1 protein expression in vivo, a reporter protein for monitoring diap1 turnover, PRAP (pre-apoptosis signal-detecting probe, based on diap1 degradation) was developed. Live-imaging analysis of PRAP revealed that diap1 degradation is regulated in a cell lineage- and stage-specific manner during *Drosophila*

sensory organ development. diap1 executes two distinct functions, one in shaft-cell survival just after the final division, and one in shaft-cell morphogenesis during its maturation stage. Both functions are exerted through the regulation of caspase activity. For the maturation stage, nonapoptotic dronc activity is required and regulated by DmIKKε. Thus, precise temporal control of the endogenous caspase inhibitor diap1 is critical for maintaining a balance between cell viability and the execution of caspases' nonapoptotic functions.

CONCLUDING REMARKS

Caspase activation that occurs on intrinsic and extrinsic stress during development and tissue regeneration supports the robust cell-fate determination of the cell community through both cell-autonomous mechanisms, such as execution of cell death or nonapoptotic functions. Caspase-mediated cell death also participates in development thorough noncell autonomous mechanisms, such as secretion of signaling molecules or force generation for neighboring cells. Thus, caspases actively participate in animal development as well as tissue regeneration.

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