Histochem Cell Biol (2006) 126:149–158
DOI 10.1007/s00418-006-0214-1

REVIEW

Cell death in development: shaping the embryo

Carlos Penaloza · Lin Lin · Richard A. Lockshin · Zahra Zakeri

Accepted: 8 June 2006 / Published online: 1 July 2006
© Springer-Verlag 2006

Abstract Cell death in animals is normally classified as type I (apoptotic), type II (autophagic) or necrotic. Of the biologically controlled types of death, in most embryos apoptosis is the most common, although in metamorphosis and in cells with massive cytoplasm type II is often seen, and intermediate forms are seen. For vertebrate embryos other than mammals, apoptosis is not seen prior to gastrulation but thereafter is used to sculpt the organs of the embryo, while overproduction of cells with subsequent death of excess cells is a common means of generating high specificity with low information cost. In zebrafish at least, the inability of embryos prior to the maternal-zygotic transition to undergo apoptosis appears to derive from the inability of the cells to resist lysis once apoptosis begins, rather than any inhibition of apoptosis. In mammalian embryos, apoptosis is seen during cavitation. Thereafter, as in other embryos, cell death plays a major role in shaping and sculpting the embryo. In those situations that have been carefully studied, cell death is under tight genetic control (including regulation of gene products whose function in cell death is not yet known, such as cdk5), with activation of apoptosis sometimes regulated by local environmental variables.

Keywords Cell death · Apoptosis · Development · cdk5 · Caspases

Histochemistry and Cell Biology Lecture presented at the 48th Symposium of the Society for Histochemistry in Stresa, Lake Maggiore, Italy, 7–10 September 2006

C. Penaloza · L. Lin · Z. Zakeri (✉)
Department of Biology, Queens College and Graduate Center of CUNY, Flushing, NY 11367, USA
e-mail: Zahra_Zakeri@qc.edu

R. A. Lockshin
Department of Biological Sciences, St. John's University, Jamaica, NY 11439, USA

Present Address:
L. Lin
Preclinical Department, Medarex Inc., Bloomsbury, NJ 08804, USA

It is now well understood that cell death is a fundamental aspect of development of almost all organisms. Historically cell death was mostly noticed in developing systems and for many years considered to be important only in development. In the last 15 years this image has changed, and the importance of cell death has been realized in many other systems and situations that govern our lives. However, the study of cell death in development still has much to teach us about the embryo and about the mechanisms of cell death. Embryonic development is dynamic and well orchestrated. Cells in very close proximity proliferate, differentiate, and die, and their decision making appears to be influenced by their microenvironment. How one cell dies whereas the neighboring cells divide is presumptively regulated by the internal and external signals that they receive. To understand how these signals are presented and recognized during development we as well as others have used many methodologies and systems. Several laboratories had to develop specific markers and in doing so learned much about the steps taken by a dying cell and how developmental cell death resembles and is different from other types of cell death.

Cell death, its types and its characterization

Cell death has been classified into three classical types: apoptosis, autophagy (also referred as lysosomal cell death), and necrosis (for review, see Zakeri and Lockshin 2002), and all three are seen during development. Both apoptosis and autophagy are considered to be under biological control and thus programmed (programmed cell death, PCD). PCD was originally defined by the demonstration of a clear physiological and biochemical sequence leading to death in a developmental situation (metamorphosis), but now the term is understood to refer to any biologically controlled and managed cell death. The different types of cell death have specific features that are used both to identify them and to characterize them. Although many features are specific the boundaries among the different types are not nearly as well defined as many assume, and there are overlapping features among the different types. It is also noteworthy that there is much crosstalk among the different organelles; this crosstalk allows autophagy to convert to apoptosis or vice versa, and it allows an apoptosis typically triggered by ligation of receptor molecules to follow the pathway of metabolically activated apoptosis. Thus if one pathway is blocked, a cell may still die by a second, highly organized and biological pathway. This result is quite common and has led to considerable confusion among researchers not attentive to the possibility.

Apoptotic PCD (Type I)

Apoptosis or type I cell death is characterized by a cell's loss of adherence to neighboring cells as well as to extracellular matrix. Morphological changes include cell rounding and condensation of nuclear material. The chromatin coalesces into one or a few masses along the nuclear membrane. PCD type I is also characterized by fragmentation of DNA to form a ladder when electrophoresed. There is release of cytochrome c and other materials from mitochondria and activation of caspases, specifically caspase 3. Cells ultimately fragment into blebs that are taken up by phagocytes. At the final stages of apoptosis, phosphatidylserine (PS) is actively extruded from the internal face of the cell membrane of the dying cell; the exteriorized PS serves as one of the markers that identify the cell as a target for phagocytosis. This type of cell death can be greatly affected by levels of ATP: if levels of energy are not sufficient, cells can undergo partial apoptosis, leading to different appearance and possibly misinterpretations. For instance, it is commonly noted that the cells of early non-mammalian embryos cannot undergo apoptosis prior to the maternal-zygotic transition (MZT). However, when zebrafish embryos are exposed to cycloheximide, both 256-cell embryos (prior to the MZT) and 1,024-cell embryos (after the MZT) activate caspase-3 after approximately 5 h. Pre-MZT embryos lyse almost immediately thereafter and thus show no morphological signs of apoptosis, whereas the older embryos persist for two more hours and develop all the signs of classical apoptosis, including phosphatidylserine exposure, DNA fragmentation, and margination of the chromatin (Negron and Lockshin 2004; J.F. Negrón and R.A. Lockshin, in press).

Lysosomal, autophagic PCD (Type II)

The appearance of large autophagic vacuoles, lysosomal derivatives that consume the bulk of the cytoplasm, is characteristic of this type of PCD. This type of cell death unlike apoptosis is not characterized by the prompt destruction of DNA. In most of the cells undergoing Type II PCD, the cytoplasm is bulky and cannot be easily shed to be phagocytosed. The function of the phagocyte is conducted within the cell. When approximately 80% of the cytoplasm has been destroyed, the condensation of the cytoplasm and coalescence and migration of the chromatin become apparent, and only then it is possible to detect by electrophoresis a DNA ladder, indicating DNA cleavage by an apoptosis-type DNase. It is not certain that the autophagy is truly a type of cell death, as opposed to the response of a cell under stress and attempting to survive by consuming its resources. Thus, as in other instances in which cells undergo autophagy, there is potential recovery, and the autophagy is a recycling mechanism, used under stressful conditions. In the documented deaths of cells by autophagy, as in insect metamorphosis, organelles are eliminated in waves. Glycogen, ribosomes, and mitochondria are eliminated in sequence. In other instances of autophagic death, such as death of prostatic epithelium following castration and possibly post-lactational involution of mammary epithelium, the actual commitment to die occurs relatively late, and cells can revert and recover if given the appropriate hormonal support. PC12 cells undergoing starvation from nerve growth factor (NGF) withdrawal can recover if the NGF is provided. They are capable of recovering until almost immediately before they lyse; this point of no return is coincident with the final autophagic destruction of mitochondria, presumptively terminating the cells' ability to maintain ionic pumps (Xue et al. 1999, 2001).

Both type I and type II cell death have been referred to as programmed cell death. Like proliferation and differentiation, cell death, especially PCD, also plays a prominent role in normal development. It is essential for the removal of unwanted cells and is critical both

for restricting cell numbers and for tissue patterning during development (Chanoine and Hardy 2003; Coucouvanis et al. 1995; Glücksmann 1951).

Necrosis

Necrosis characterized as uncontrolled death, occurs as a result of high levels of stress, such as injury. Rapid and large changes in critical ions, osmolarity, and pH lead to the lysis of the cell. In the absence of adequate mitochondrial function and effective ion pumps, the high concentration of impermeable protein as the primary intracellular anion tends to draw water in, and under anaerobic conditions lactic acid will also accumulate, pulling in more water. Ultimately the cell membrane becomes leaky or bursts, allowing the escape of cell contents. In most situations, the uncontrolled release of cell contents activates an inflammatory response, including the invasion of mast cells, phagocytes, and NK cells. Massive necrosis can for many reasons be extremely threatening to an organism and, in higher vertebrates, the leakage of intracellular materials can lead to autoimmune reactions. Thus, the provocation of inflammation is an important, although derivative, distinction between necrosis and apoptosis. In fact, there is evidence that the ingestion of apoptotic bodies suppresses an inflammatory response (Edinger and Thompson 2004; Haslett et al. 1994; Lockshin et al. 2001; Schmied et al. 1993). In contrast to the obvious osmotic mechanisms that lead to the swelling and rupture of necrotic cells, the shrinkage of apoptotic cells was a mystery for a long time (Lockshin and Beaulaton 1981). It now appears that the apoptotic cells shrink by losing intracellular K^+ during a period in which sufficient energy is still available to run pumps (Bortner and Cidlowski 2002). Thus, a key distinction between apoptosis and necrosis is the availability of intracellular energy. It is not surprising to encounter situations in which cells begin to undergo apoptosis but ultimately lyse in a necrotic fashion. One such situation is seen when hepatotoxins are given to intact animals; at the periphery of the acutely toxic zone, some cells initiate apoptosis but ultimately fail and undergo necrosis (Ledda-Columbano et al. 1991). Of course, when cell death is activated in cultured cells, necrosis is the ultimate fate of the apoptotic fragments, since no phagocytes are present.

Cell death during development is common to all examined embryos

It was a belated though intellectually necessary realization that the apparently wasteful process of cell death in early embryos is a solution to the problem of how much genetic information would be needed to construct an animal. To take an easily understood image, the quantity of information to connect 1,000 neurons to 1,000 specific targets will vary according to several assumptions, but even in the simplest version, the information required would be immense. It is surprising but mathematically demonstrable that much less genetic information (DNA sequences) is needed to produce an excess number of presynaptic neurons that can generally and with little requirement for specificity find their way to the target area, and to allow only those that make successful synapses to survive. Similarly, to randomly reconfigure DNA to generate a huge variety of cells, each making a different immunoglobulin, and to kill off those that are inappropriate, requires only approximately 60 genes, as opposed to the 100,000 or so that would be necessary to code individually for all the antibodies a mammal is capable of producing.

While these two examples are spectacular, a similar logic can be deduced for much that is common in embryonic development, including regionalization, pattern formation, limb and appendage configuration, development of axes, and response to location along an axis. Thus, it is not surprising to find that the cell death as a mechanism is highly conserved among a wide variety of embryos and metamorphosing organisms (Glücksmann 1951). In *C. elegans*, death of 131 of the original 1,090 cells leaves an adult with 959 somatic cells. This system has been instrumental in elucidating genes involved in different pathways of cell death (Horvitz et al. 1994). In plants, cell death plays an important role in a variety of aspects from fertilization through development as well as in response to environmental insults. For example, PCD is involved in xylogenesis, aerenchyma formation, petal senescence, endosperm development, hypersensitive response, and various forms of abiotic stress (for review, see Hoeberichts and Woltering 2003). Although cell death in plants sometimes resembles apoptosis in many facets, the plant cells are enclosed in rigid cell walls and cannot round up or fragment. Thus, the unique cell death found in plants depends on vacuolar lytic function, and dying cells display many functional vacuoles (Fukuda 2000; Kuriyama and Fukada 2002). Furthermore, plants process oxygen in both mitochondria and chloroplasts, and therefore reactive oxygen species (ROS) are important signals in the activation of plant PCD. For instance, in soybean cells, PCD can be triggered by ROS (Solomon et al. 1999). Increasing evidence indicates that the plant PCD proceeds through a cell death mechanism that is functionally conserved between animals and plants (for review, see Hoeberichts and Woltering 2003).

Cell death is known in invertebrates such as insects (Abrams et al. 1993; Steller and Grether 1994) and sea urchins (Yüce and Sadler 2001). There is considerable cell death in *Drosophila* embryos, occurring in the central nervous system, similar to vertebrate embryos, and elsewhere (Abrams et al. 1993). As in *Caenorhabditis*, these deaths are all controlled by a limited number of genes, although unlike *Caenorhabditis*, the insect genes appear neither to be proteases nor related to mammalian death control sequences. A set of three genes, named reaper, grim, and hid, are found within the 75C1,2 region of the third chromosome (Chen et al. 1996; Grether et al. 1995; White et al. 1994). Knocking out these genes blocks all cell deaths in the embryo. Similarly, as judged from appropriate knockouts and knockins, these genes are required for the death of ommatidial progenitors in eyeless and other mutants. The deaths are presumed to be apoptotic, though they have not been examined in great morphological detail. When *Drosophila* cells are cultured, an apoptosis sequence can be easily demonstrated, although the proteases involved are much less closely related to the caspases of either *Caenorhabditis* or vertebrates than is, for instance, the *Caenorhabditis* caspase ced-3 to human caspases. Furthermore, although there have been various indirect measurements suggesting caspase-like enzymes in metamorphosis, these have not been well documented. In fact, when attempting to document proteases in insect metamorphosis by substrate preference and cleavage site or susceptibility to inhibitors, one cannot easily identify a caspase. The bulk of the proteolysis during metamorphosis is conducted by other enzymes, such as lysosomal cathepsins, calpain, or proteasomal proteases (C.O. Facey and R.A. Lockshin, in preparation; Jochová et al. 1997a, b; Schwartz et al. 1990). Perhaps this is because metamorphic cell death is predominantly autophagic (see earlier). If insect caspases are activated during the final phases of metamorphic cell death, when the cells finally become TUNEL and PS positive and contain DNA fragmented at nucleosomes, the enzymes are not well documented.

In lower vertebrates there is substantial cell death during early embryogenesis. Some of this death has been characterized in zebrafish and established as important for such developmental processes as forming the olfactory pits, separating the lens from the cornea, opening the anus, shaping the fins, establishing the smoothness of the skin, and sculpting and modeling the brain and central nervous system. A cell death-like process eliminates the organelles from lens cells. Large Rohon-Beard neurons form in each segment only to die at a slightly later stage (Coen et al., 2001; Svoboda et al. 2001; Williams et al. 2000). Interference with cell death by blocking caspase activity produces several distinct abnormalities, most notably in the notochord and in skeletal and CNS development (N. Abraham and R.A. Lockshin, in preparation; Cole and Ross 2001). These abnormalities are different from those produced in mammals by knocking out apoptosis-promoting genes, such as the initiator or apical protease caspase-9 or apoptosis-activating factor 1 (Apaf-1). However, studies in the zebrafish have been done by administration of inhibitors rather than knockout, and it is not certain that the comparisons are valid.

Cell death is an early event during mammalian development

Mammalian embryos differ from other vertebrate embryos in many ways, but for our purposes a central issue is compaction, cavitation, and formation of the inner cell mass. This deviation from normal vertebrate development to form a placenta is also characterized by an extremely early activation of zygotic genes. Likewise, cell death starts at a very early stage in mammalian development. While in most vertebrate embryos one sees no cell death prior to gastrulation, in mammalian embryogenesis cell death begins as early as inner cell mass differentiation in blastocysts (Hardy et al. 1989; for review, see Spanos et al. 2002) (Fig. 1a–c). We used the PS to identify cell death in the preimplantation developing embryo. As described earlier, the phosphatidylserine is a phospholipid normally asymmetrically expressed in the inner leaflet of the plasma membranes in living cells. During apoptotic cell death, due to the loss of membrane potential, these molecules are actively translocated to the outer leaflet of the plasma membrane. Annexin V is a very specific apoptotic marker since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline. One can detect apoptotic cells as fluorescent cells against a dark background. Staining is visualized by fluorescence or confocal microscopy or by fluorescence activated cell sorting.

As early as the two-cell stage we can find cell death in the polar bodies. We did not find dying cells in embryos containing 1 to 8 cells, but a few cells die between the 16-cell and blastula stages. We also showed that generalized inhibitors of caspases affect normal development even at stages where there was no cell death (Fig. 1). Inhibition of caspase activity led to the arrest of embryonic development. This cell death in the developing pre-implantation embryo was not caspase

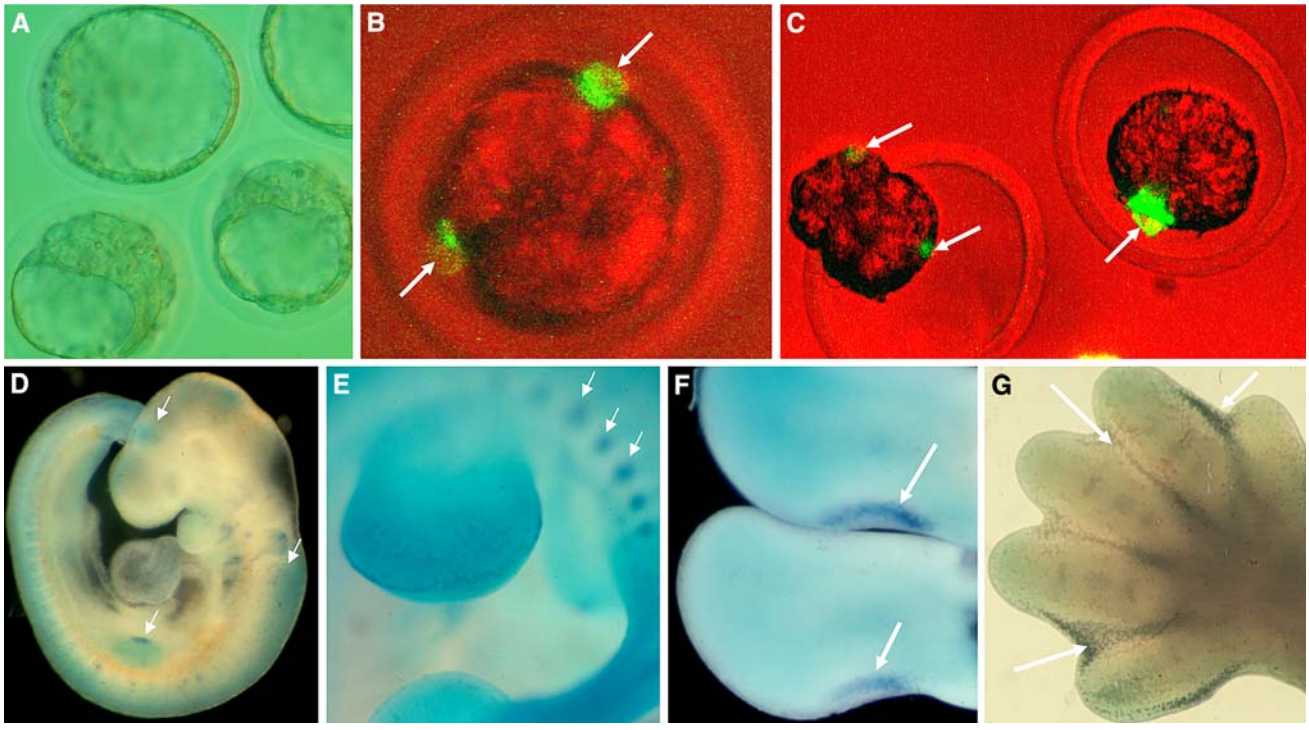

Fig. 1 Cell death in developing mouse embryos. **a** Cultured mouse embryos at the midblastula and expanded blastula stages observed with light microscopy. **b**, **c** Detection of phosphatidylserine by Annexin V on dying cells during normal development. The polar bodies exteriorize phosphatidylserine by the two-cell stage. At compaction and in early blastula, dead cells are present and at expanded blastula, cell death is seen where the inner cell mass meets the trophectoderm. Either one or two dead cells are seen at this time. When there are two dead cells, they are at opposite poles at the same latitude. Nile blue sulfate staining of dying cells (*arrows*) in ED9.5 (**d**), ED10.5 (**e**), and ED11.5 (**f**) mouse embryos. **g** Nile blue sulfate staining of dead cells (actually, vacuoles of phagocytes) in the interdigital regions of a 13.5 day embryonic mouse hand palette. Some of the most prominent regions are indicated by *arrows*

dependent. Caspase activation normally induces cell death, but under the conditions used, caspase inhibition led to further cell death and embryonic development inhibition as a result (Zakeri et al. 2005).

A major part of organogenesis involves cell death

A crucial role for cell death is seen during organogenesis and tissue remodeling. Cell death was originally noticed in metamorphosing animals. Cell death destroys almost all larval tissues during metamorphosis of insects and amphibians (Fox 1973). In *Drosophila*, cell death is induced in larval midgut by the steroid hormone ecdysone (Lee et al. 2002). In tadpoles, regression of the tail and destruction of the gills are attributed to tightly controlled cell death (Jeffery 2002). Precisely regulated cell death contributes to the formation of different body parts and multiple organs of an organism. In developing rat lens, apoptosis removes the nuclei in the process termed nuclear death, and leaves the rest of the cells remaining functional (Gao et al. 1997; Gupta et al. 2002; Wu and Ding 2002). It has been shown that the development of the rat lens vesicle involves apoptotic elimination of the cells between the surface ectoderm and the optic vesicle to help trigger invagination and facilitate separation from the ectoderm. Apoptosis also aids in the bowing of the optic vesicle during lens invagination (Mohamed and Amemiya 2003). Apoptotic cell deaths shape the future inner ear structure in day 5 chick embryo (Avallone et al. 2002). In cardiac morphogenesis, cell death is essential in generating the overall four-chambered architecture of the heart (Abdelwahid et al. 2002). The role of cell death in muscle development has been well studied. In moths, muscle cell death is required for correct muscle patterning (Fahrbach et al. 1994; Haas et al. 1995). In rat skeletal muscle, cell death persists during the first three post-natal weeks, suggesting an indispensable role for cell death in the development of skeletal muscle (de Torres et al. 2002). In *Xenopus* cell death destroys all the primary myotomal myofibers, which are replaced progressively by secondary "adult" multi-nucleated myofibers in the construction of muscles during development (Chanoine and Hardy 2003).

Cell death sculpts the limbs of all amniotes, such as humans, mouse, and birds by removal of interdigital webs (Ballard and Holt 1968; Zakeri and Ahuja 1994; Hurle and Colombatti 1996). Cell death in limb development begins at embryonic gestation day (ED) 10.5 and peaks at ED14.5, at which stage there is enough cell death to permit cellular and molecular analysis (Fig. 1d–g). Cell death can be observed in the anterior and posterior marginal zones of the developing limb bud and in later stages in almost all of the interdigital mesenchymal tissue. Cell viability in these regions is dependent on the condensation of core mesenchymal cells from the foot or hand palette. In the absence of this condensation, as is the case in interdigital area, cells undergo apoptosis. This cell death accompanies the formation of free and independent digits of birds and mammals. Cell death can be recognized in the embryo by several techniques. One more effective way is in vivo detection of cell death using vital dye staining. One can take advantage of the fact that a characteristic of cell death is the increase in permeability of cell membranes, allowing selective permeability to specific vital dyes, such as Nile blue sulfate, which enters the cell and localizes into the acidic compartments of the cells such as the lysosomes. We and others have used this type of staining for identification of cell death in the developing limbs of mouse embryos (Fig. 1d–g). One of the best ways to identify and even more specifically examine the type of cell death is to use electron microscopy. Using this method we found that there are different types of cell death in the dying interdigital regions of the limbs. We find that most cell death is apoptotic in nature with the classical margination of nuclei and rounding of the cells, which are often seen as apoptotic bodies engulfed by macrophages (Fig. 2a, d). Although this type of cell death is the major type found we occasionally also find necrotic cell death (Fig. 2b) and also cells resembling autophagic cell death (Fig. 2c).

One of the characteristics of apoptotic cell death is engulfment by macrophages. We have taken advantage of this fact and asked if antibodies specific for mature macrophages can recognize the phagocytic cells in these regions. Although mature macrophages do not circulate until later during development we can detect phagocytic cells in the area of the limb cell death as early as our first detection of the dying cell, i.e. ED 10.5 and more intensely at ED 13.5 (Fig. 3a, b). The presence of these cells is most likely indicative of differentiation of the mesenchymal cells to phagocytic cells expressing macrophage-like cell surface proteins. In addition these phagocytic cells are very active in lysosomal activity as measured by activation of acid phosphatase (Zakeri et al. 1994). The activation of acid phosphatase can also be found in some of the individual dying cells (Fig. 3c), leading us to infer that maybe type II cell death is taking place in the cells that are not found in the macrophages.

A hallmark of apoptotic cell death is the fragmentation of DNA to distinct ladders. Although this can be easily seen using gel electrophoresis of in vitro cultured

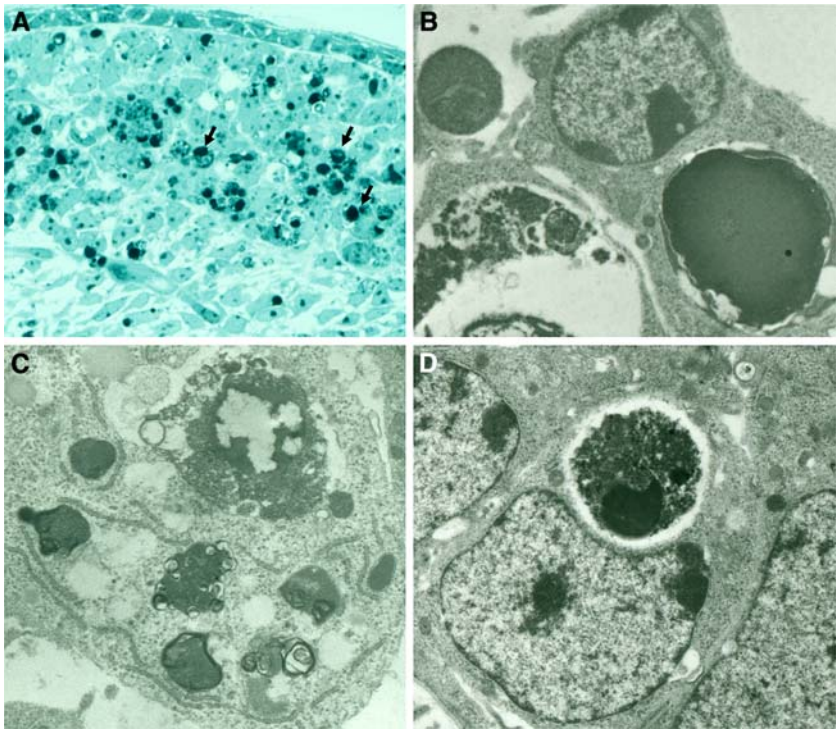

Fig. 2 Cell death examined by electron microscopy. **a** Thin section of hand palette showing apoptotic bodies within macrophages. **b–d** Electron micrographs showing different forms of cell death (**b**) necrotic cell death (left) with disorganized nucleus and cytoplasm and (right) phagocytosed apoptotic body (**c**) autophagic cell death with large autophagic vacuoles (**d**) phagocytosed apoptotic body with condensed nucleus and cytoplasm that is niether extracted nor precipitated

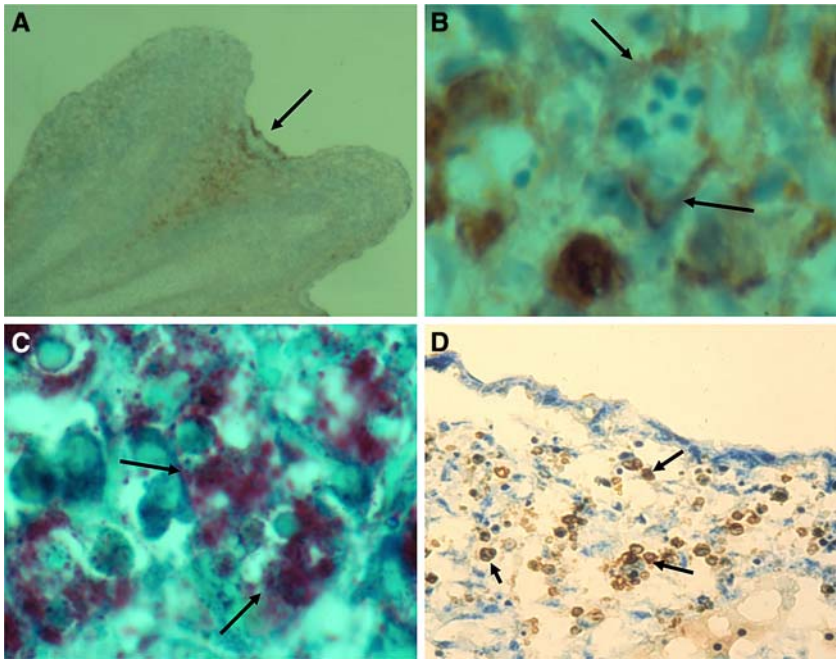

Fig. 3 Detection of phagocytic cells in developmental cell death. **a** Low magnification of the interdigital area of limb stained for phagocytic cells (*dark brown*) by antibody F4/80. **b** High magnification of (**a**), *arrows* show the phagocytic cells with apoptotic bodies. **c** Stained section of interdigital region of the limb for the activity of lysosomes. Activity of acid phosphatase in lysosomes indicated by red precipitate (arrows). **d** Detection of DNA fragmentation using TUNEL/DAB-peroxidase reaction. Dead cells (*arrows*) are revealed in the interdigital area of mouse limb

cells, finding it in a situation where only a few cells are dying at a time is more challenging. However, one can use the end labeling TUNEL to identify the dying cells. This technique is more applicable to the developing embryo, since one can see the fragmented DNA at the cellular level without disrupting the cellular arrangement, so one can measure the levels of cell death very sensitively and with spatial specificity. TUNEL is an acronym for terminal deoxyuridine nucleotide end labeling, and its method of action is by labeling any free 3′ end of DNA. This method detects fragmented DNA from necrotic, mitotic as well as apoptotic cells, but the level of DNA fragmentation is so much greater in apoptotic cells that this technique is among the most reliable assays for apoptotic cell detection. The fragmented DNA within the dying cell is usually visualized as a dark red to brown staining in the cell. The downside to using this technique is that only late-stage apoptotic cells can be reliably scored. Commercial kits are readily available for this assay. By this measure we can find many TUNEL positive cells (Fig. 3d) in correlation with the regions of high positive signal for macrophage staining and acid phosphatase activity (Fig. 3c). By all these measurements we can monitor the occurrence and type of cell death during the development of the limb.

Cell death sets up the correct patterning of the embryo and is under genetic regulation

During development, cell death plays an important role in the elimination of redundant cells and thus determines the patterning of structures in both vertebrates and invertebrates. Precise patterns of the cell death are critical for the development of a number of different organs including the nervous system and the immune system. During vertebrate brain development, 20 to 80% of the neurons originally produced are eliminated by cell death (Gordon 1995), and more than 80% of ganglion cells in the cat retina die shortly after birth (Barres and Raff 1999). Much of the cell death is due to limited quantities of trophic factors, such as NGF; the only neurons that survive are those supplied with NGF (Vyas et al. 2002). In the human immune system, about 95% of developing B cells will die due to faulty gene rearrangement, anti-self receptor expression, or lack of stimulation, and immunological tolerance is achieved through highly controlled cell death (Osborne 1998; Gercel-Taylor et al. 2002). Cell death is also a pivotal part to the development of the germ cells; in human females, 80% of the original germ cells (oocytes) die by apoptosis by the time of birth (for review, see Reynaud and Driancourt 2000).

Cell death in the embryo has been studied genetically for the most part, with some pioneering observations with mutations found that lead to limb malformations, such as Dominant hemimelia (Dh), Luxoid (Lu), Luxate (Lx), Oligosyndactyly (O), Extra toes (Xt), and Hammertoe (Hm). The Hm mutants were used by us to evaluate the relationship between the altered pattern of limb formation with cell death. The Hm mice have webbing between digits 2, 3, 4, and 5. We first identified the pattern of cell death in normal, heterozygote and homozygote embryos during different

times of gestation from day 11.5 to 15.5. The homozygous normal embryo has the correct pattern of cell death, which is partially altered in heterozygotes and completely altered in the homozygous mutant. In the homozygous mutant hand at day 14.5 when we find the peak of cell death in the interdigital webs we find cell death only between digit one and two and no cell death between the other digits (Fig. 4a). This lack of cell death reflects the phenotype seen in the adult where there is webbing between all the digits except between digit one and two (Fig. 4b). This aberrant pattern of cell death does not have any effect on the correct pattern of skeleton formation (Fig. 4c) and thus is reflective of soft tissue syndactyly. More interestingly we can correct this genetic abnormality by exposing the embryo in utero to all *cis*-retinoic acid (RA) at a specific window of development and cause the rescue of the malformation. In this case the homozygous mutant embryos are born with correct limb patterning since we have induced cell death at the correct time by RA. These findings indicate a direct correlation between pattern formation in the limb and correct level and pattern of cell death.

From genetic studies using *C. elegans* as well as *Drosophila* and mice it is obvious that developmental cell death is under genetic regulation. The presence of a number of mutant animals with altered levels of cell death gives credence for the regulation of cell death by specific gene activity. Some of the most important studies crucial for our understanding the mechanism of apoptosis came from studies of *C. elegans* in measuring embryonic or developmental cell death, which led to the well known bcl-2 like molecules, normally inhibiting cell death, activation of caspase-like molecules destroying the cells, and the existence of "eat me" signals on the surface of apoptotic cells. The majority of the information known about cell death in the embryo came from manipulation of genes and characterization of the resulting phenotypes. Further genetic analysis of cell death will very likely provide much more information, which may lead to further understanding of cell death in development.

One of the sets of genes that may be instrumental in regulating cell death is the cell cycle related genes. One particularly interesting group is that of cyclin dependent kinases. We found that a unique member of this family i.e. cyclin dependent kinase 5 (cdk5), identified by its homology to cdc2 and recognized by its activation during neuronal proliferation, also has an activity during cell death (for review, see Dhavan and Tsai, 2001). When antibodies specific to Cdk5

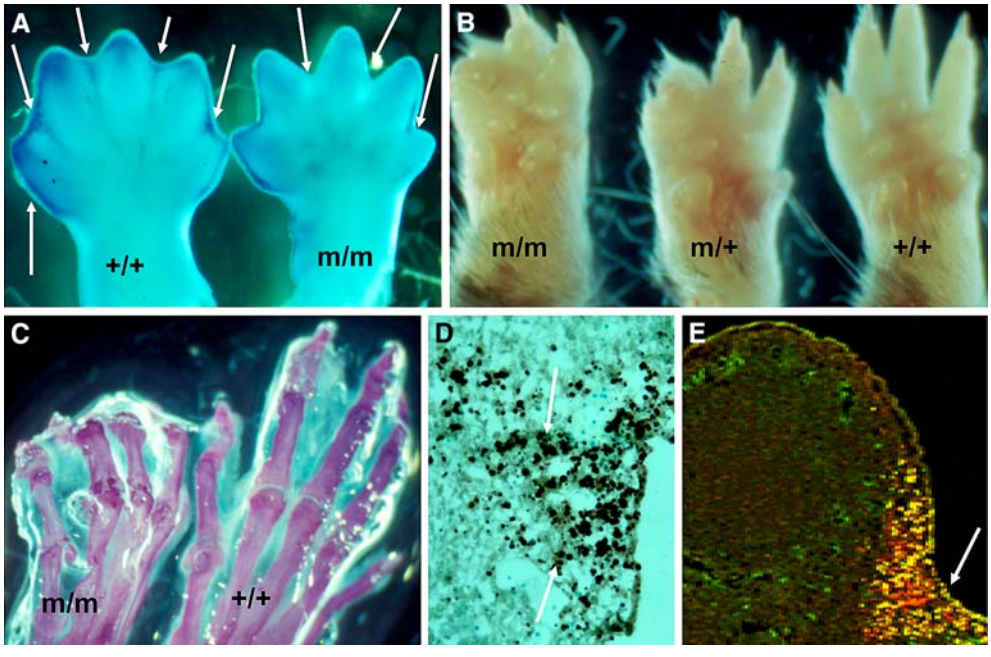

Fig. 4 Expression of cell death-related genes in development. **a** Cell death (*arrows*) in interdigital areas of normal developing mouse limb (+/+), and significantly less cell death (*arrows*) in the same region of hammertoe mutant (m/m). **b** Hammertoe heterozygous (m/+) and homozygous (m/m) show no separation between digits 2 and 5 and increased curvature, while there is complete separation between digits 2 and 5 in wild type mice (+/+). **c** Skeletal staining of hammertoe mutants, showing increase in curvature of digits in (m/m) hammertoe mice as opposed to the wild type mice (+/+). **d** Cdk5 expression (*dark spots pointed by arrows*) by immunohistochemistry in interdigital area of ED13.5 mouse limb. **e** Confocal merge image of red Cy-3 labeling (Cdk5 expression) and green FITC labeling (DNA fragmentation). The *yellow signal* indicates co-expression of Cdk5 and DNA fragmentation in ED13.5 mouse developing limb

were used we found a diffuse level of expression in differentiating neurons but an intense signal in dying cells of the hand plate in the interdigital regions (Fig. 4d). This expression correlated with the cells positive for DNA fragmentation by TUNEL and when confocal images were merged, many cells showed both signals (Fig. 4e). The expression of the cdk5 is not unique to the dying cells of the developing limb; its expression is found in all dying cells seen in different parts correlating with different organs of the developing embryo (Zhang et al., 1997). We found that the expression in the dying cells correlates with the post-translational increase in kinase activity and is not due to an increase in transcription or translation of cdk5 (Zhu et al., 2002). Additionally inducing cell death in the day 10.5 embryo with cyclophosphamide (CP), which leads to 90% cell death, we isolated using both yeast two hybrid and pulldown assay a cyclin like interacting protein (p35) that appears to be cleaved by a calpain during cell death to p25 and to interact and activate CDK5 only during cell death (Zhu et al., 2002). Interestingly the activation of CDK5 does not require caspase, functional mitochondria-related bin, or p53 (Lin et al., 2006; Ye et al., in preparation). We can conclude that the activity of CDK5 during differentiation is regulated by a larger interacting activating protein p35; and during activation of cell death activated calpains cleave the p35 to p25, which interacts and activates CDK5 which then acts to propagate cell death. From our studies using dominant negative and siRNA and anti-sense RNA we can conclude that CDK5 activation is not the inducer of cell death, but that its activation is a result of cell death. Since it is seen in a number of situations in which the cells die by different ways or triggering events its activation is a downstream event regulating the process of cell death.

In summary cell death is an instrumental feature of development, without which one cannot have an embryo and therefore continuation of a species. The incidence of this cell death is uniquely regulated and controlled by specific genetic programs that can be somewhat manipulated by environmental factors. Developmental cell death represents a special type of cell death with common features with many other situations in which we see cell death and special features only seen in development.

Acknowledgements The authors wish to thank Drs. Yong Zhu and Daniela Quaglino for their technical assistance. We would also like to thank NYC-LSAMP and MAGNET-AGEP, sponsored by NSF and the National Institutes of Health (MARC Minority Access to Research Careers) GM 070387-01 for their support.

References

Abdelwahid E, Pelliniemi LJ, Jokinen E (2002) Cell death and differentiation in the development of the endocardial cushion of the embryonic heart. Microsc Res Tech 58(5):395–403

Abrams JM, White K, Fessler LI, Steller H (1993) Programmed cell death during *Drosophila* embryogenesis. Development 117:29–43

Avallone B, Balsamo G, Trapani S, Marmo F (2002) Apoptosis during chick inner ear development: some observations by TEM and TUNEL techniques. Eur J Histochem 46(1):53–59

Ballard KJ, Holt SJ (1968) Cytological and cytochemical studies on cell death and digestion in the foetal rat foot: the role of macrophages and hydrolytic enzymes. J Cell Sci 3:245–262

Barres BA, Raff MC (1999) Axonal control of oligodendrocyte development. J Cell Biol 147:1123–1128

Bortner CD, Cidlowski JA (2002) Apoptotic volume decrease and the incredible shrinking cell. Cell Death Differ 9:1307–1310

Chanoine C, Hardy S (2003) Xenopus muscle development: from primary to secondary myogenesis. Dev Dyn 226(1):12–23

Chen P, Nordstrom W, Gish B, Abrams JM (1996) Grim, a novel cell death gene in Drosophila. Genes Dev 10(14):1773–1782

Coen L, du Pasquier D, Le Mevel S, Brown S, Tata J, Mazabraud A (2001) Xenopus Bcl-X(L) selectively protects Rohon-Beard neurons from metamorphic degeneration. Proc Natl Acad Sci USA 98:7869–7874

Cole LK, Ross LS (2001) Apoptosis in the developing zebrafish embryo. Dev Biol 240:123–142

Coucouvanis ED, Martin GR, Nadeau JH (1995) Genetic approaches for studying programmed cell death during development of the laboratory mouse. Methods Cell Biol 46:387–440

Dhavan R, Tsai LH (2001) A decade of CDK5. Nat Rev Mol Cell Biol 2(10):749–759

Edinger AL, Thompson CB (2004) Death by design: apoptosis, necrosis and autophagy. Curr Opin Cell Biol 16(6):663–669

Fahrbach SE, Choi MK, Truman JW (1994) Inhibitory effects of actinomycin D and cycloheximide on neuronal death in adult Manduca sexta. J Neurobiol 25(1):59–69

Fox H (1973) Ultrastructure of tail degeneration in *Rana temporaria* larvae. Folia Morphol (Prague) 21:109–112

Fukuda H (2000) Programmed cell death of tracheary elements as a paradigm in plants. Plant Mol Biol 44(3):245–253

Gao CY, Zakeri Z, Zhu Y, He H, Zelenka PS (1997) Expression of Cdk5, p35, and Cdk5 associated kinase activity in the developing rat lens. Dev Genet 20:267–275

Gercel-Taylor C, O'Connor SM, Lam GK, Taylor DD (2002) Shed membrane fragment modulation of CD3-zeta during pregnancy: link with induction of apoptosis. J Reprod Immunol 56(1–2):29–44

Glücksmann A (1951) Cell deaths in normal vertebrate ontogeny. Biol Rev Camb Philos Soc 26:59–86

Gordon N (1995) Apoptosis (programmed cell death) and other reasons for elimination of neurons and axons. Brain Dev 17:73–77

Grether ME, Abrams JM, Agapite J, White K, Steller H (1995) The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev 9(14):1694–1708

Gupta A, Tsai LH, Wynshaw-Boris A (2002) Life is a journey: a genetic look at neocortical development. Nat Rev Genet 3(5):342–355

Haas AL, Baboshina O, Williams B, Schwartz LM (1995) Coordinated induction of the ubiquitin conjugation pathway accompanies the developmentally programmed death of insect skeletal muscle. J Biol Chem 270(16):9407–9412

Hardy K, Handyside AH, Winston RM (1989) The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development 107:597–604

Haslett C, Savill JS, Whyte MKB, Stern M, Dransfield I, Meagher LC (1994) Granulocyte apoptosis and the control of inflammation. Philos Trans R Soc Lond B Biol Sci 345:327–333

Hoeberichts FA, Woltering EJ (2003) Multiple mediators of plant programmed cell death: interplay of conserved cell death mechanisms and plant-specific regulators. Bioessays 25(1):47–57

Horvitz HR, Shaham S, Hengartner MO (1994) The genetics of programmed cell death in the nematode *Caenorhabditis elegans*. Cold Spring Harb Symp Quant Biol 59:377–386

Hurle JM, Colombatti A (1996) Extracellular matrix modifications in the interdigital spaces of the chick embryo leg bud during the formation of ectopic digits. Anat Embryol (Berl) 193(4):355–364

Jeffery WR (2002) Programmed cell death in the ascidian embryo: modulation by FoxA5 and Manx and roles in the evolution of larval development. Mech Dev 118(1–2):111–124

Jochová J, Quaglino D, Zakeri Z, Woo K, Sikorska M, Weaver V, Lockshin RA (1997a) Protein synthesis, DNA degradation, and morphological changes during programmed cell death in labial glands of *Manduca sexta.* Dev Genet 21:249–257

Jochová J, Zakeri Z, Lockshin RA (1997b) Rearrangement of the tubulin and actin cytoskeleton during programmed cell death in *Drosophila* salivary glands. Cell Death Differ 4:140–149

Kuriyama H, Fukada H (2002) Developmental programmed cell death in plants. Curr Opin Plant Biol 5(6):568–573

Ledda-Columbano GM, Coni P, Curto M, Giacomini L, Faa G, Oliverio S, Piacentini M, Columbano A (1991) Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide. Am J Pathol 139:1099–1109

Lee CY, Cooksey BA, Baehrecke EH (2002) Steroid regulation of midgut cell death during Drosophila development. Dev Biol 250(1):101–111

Lin L, Ye YX, Zakeri Z (2006) p53, Apaf-1, caspase-3, and -9 are dispensable for Cdk5 activation during cell death. Cell Death Differ 13(1):141–150

Lockshin RA, Beaulaton J (1981) Cell death: questions for histochemists concerning the causes of the various cytological changes. Histochem J 13:659–666

Lockshin RA, Facey CO, Zakeri Z (2001) Cell death in the heart. Cardiol Clin 19:1–11

Mohamed YH, Amemiya T (2003) Apoptosis and lens vesicle development. Eur J Ophthalmol 13(1):1–10

Negron JF, Lockshin RA (2004) Activation of apoptosis and caspase-3 in zebrafish early gastrulae. Dev Dyn 231:161–170

Osborne BA (1998) Apoptotic signaling in lymphocytes. In: Lockshin RA, Zakeri Z, Tilly JL (eds) When cells die: a comprehensive evaluation of apoptosis and programmed cell death. Wiley-Liss, New York, pp 245–266

Reynaud K, Driancourt MA (2000) Oocyte attrition. Mol Cell Endocrinol 163:101–108

Schmied M, Breitschopf H, Gold R, Zischler H, Rothe G, Wekerle H, Lassmann H (1993) Apoptosis of T lymphocytes in experimental autoimmune encephalomyelitis: evidence for programmed cell death as a mechanism to control inflammation in the brain. Am J Pathol 143:446–452

Schwartz LM, Myer A, Kosz L, Engelstein M, Maier C (1990) Activation of polyubiquitin gene expression during developmentally programmed cell death. Neuron 5:411–419

Solomon M, Belenghi B, Delledonne M, Menachem E, Levine A (1999) The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11(3):431–444

Spanos S, Rice S, Karagiannis P, Taylor D, Becker DL, Winston RM, Hardy K (2002) Caspase activity and expression of cell death genes during development of human preimplantation embryos. Reproduction 124(3):353–63

Steller H, Grether ME (1994) Programmed cell death in Drosophila. Neuron 13:1269–1274

Svoboda KR, Linares AE, Ribera AB (2001) Activity regulates programmed cell death of zebrafish Rohon-Beard neurons. Development 128:3511–3520

de Torres C, Munell F, Roig M, Reventos J, Macaya A (2002) Naturally occurring cell death during postnatal development of rat skeletal muscle. Muscle Nerve 26(6):777–783

Vyas S, Bechade C, Riveau B, Downward J, Triller A (2002) Involvement of survival motor neuron (SMN) protein in cell death. Hum Mol Genet 11(22):2751–2764

White K, Grether ME, Abrams JM, Young L, Farrell K, Steller H (1994) Genetic control of programmed cell death in Drosophila. Science 264:677–683

Williams JA, Barrios A, Gatchalian C, Rubin L, Wilson SW, Holder N (2000) Programmed cell death in zebrafish rohon beard neurons is influenced by TrkC1/NT-3 signaling. Dev Biol 226:220–230

Wu GS, Ding Z (2002) Caspase 9 is required for p53-dependent apoptosis and chemosensitivity in a human ovarian cancer cell line. Oncogene 21(1):1–8

Xue L, Fletcher GC, Tolkovsky AM (1999) Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. Mol Cell Neurosci 14:180–198

Xue L, Fletcher GC, Tolkovsky AM (2001) Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. Curr Biol 11:361–365

Yüce Ó, Sadler KC (2001) Post-meiotic unfertilized starfish eggs die by apoptosis. Dev Biol 237:29–44

Zakeri Z, Ahuja HS (1994) Apoptotic cell death in the limb and its relationship to pattern formation. Biochem Cell Biol 72:603–613

Zakeri Z, Lockshin RA (2002) Cell death during development. J Immunol Methods 265(1–2):3–20

Zakeri Z, Quaglino D, Ahuja HS (1994) Apoptotic cell death in mouse limb and its suppression in the hammertoe mutant. Dev Biol 165:294–297

Zakeri Z, Lockshin RA, Criado-Rodriguez LM, Martinez-A C (2005) A generalized caspase inhibitor disrupts early mammalian development. Int J Dev Biol 49(1):43–47

Zhang Q, Ahuja HS, Zakeri Z, Wolgemuth DJ (1997) Cyclin-dependent kinase 5 is associated with apoptotic cell death during developmental and tissue remodeling. Dev Biol 183:222–233

Zhu Y, Lin L, Kim S, Quaglino D, Lockshin RA, Zakeri Z (2002) Cyclin dependent kinase 5 and its interacting proteins in cell death induced in vivo by cyclophosphamide in developing mouse embryos. Cell Death Differ 9(4):421–430