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DEVELOPMENTAL REGULATION OF APOPTOSIS IN ZEBRAFISH OOCYTES, 64-CELL STAGE BLASTOMERES, AND GERM-RING STAGE EMBRYOS

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ABSTRACT

DEVELOPMENTAL REGULATION OF APOPTOSIS IN ZEBRAFISH OOCYTES, 64-CELL STAGE BLASTOMERES, AND GERM-RING STAGE EMBRYOS

Javier Francisco Negrón

The zebrafish early embryo acquires a general capability for apoptosis as a consequence of passage through the maternal-zygotic transition, the time that marks the beginning of zygotic transcription and the degradation of inherited maternal factors. We describe in detail the origin and regulation of the programmed cell death machinery found in the zebrafish early embryo. To elicit an apoptotic response from zebrafish early embryos, gastrulating germ-ring stage embryos were chronically exposed to the protein synthesis inhibitor cycloheximide. Chronic exposure of germ-ring stage embryos to cycloheximide resulted in the rapid activation of an apoptotic response by the embryos, which was evident by the presence of extensive DNA fragmentation, chromatin condensation, and caspase-3 activation.

Zebrafish eggs synchronously die if attempted fertilization is unsuccessful. The lack of fertilization results in the elimination of the eggs through apoptosis within eight hours, a time equivalent to mid-gastrulation in fertilized control embryos. The presence of extensive cytoplasmic

fragmentation and the activation of caspase-3 by the egg provides evidence that the death is apoptotic. In addition, if 64-cell stage embryos are chronically exposed to cycloheximide, the embryo enters cell cycle arrest, and fails to undergo classical apoptosis, yet dies via a caspase-3 mediated type of secondary necrosis within five hours, a time equivalent to mid-gastrulation in control embryos.

The data reported here generate support for the hypothesis that the zebrafish egg is supplied with the machinery for apoptosis prior to fertilization, and that if fertilization occurs, the formation of the zygote state activates an inhibition of the apoptotic machinery that persists until mid-gastrulation. These maternal inhibitors are effective at preventing classical apoptosis until the embryo reaches the maternal-zygotic transition. Once the maternal-zygotic transition begins, the embryo regains the ability to undergo classical apoptosis as demonstrated by the apoptotic death of germ-ring stage embryos upon exposure to cycloheximide. These results lead us to believe that post-maternal-zygotic transition embryos have the capability to initiate activation of the apoptotic response without the need for *de novo* protein synthesis. Taken together, our data reveal an elaborate mechanism of cell suicide, which has its roots in the unfertilized egg.

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CHAPTER I

Introduction

During the development of the zebrafish (*Danio rerio*) early embryo, dynamic changes in cell number and shape occur without the death of a single cell until mid-gastrulation, approximately eight hours post-fertilization. It has been proposed that every cell expresses the machinery for programmed cell death (PCD), and that all cells except blastomeres require signals from other cells to avoid PCD (Raff *et al.*, 1993; Ishizaki *et al.*, 1995; Weil *et al.*, 1996). A variety of mammalian cell types require survival factors to keep the death program suppressed. Mammalian nucleated cells constitutively express all the effector proteins required for apoptosis. Activation of the cell death machinery may depend on *de novo* mRNA and protein synthesis. Therefore anything that blocks these intracellular signaling pathways should induce apoptosis.

Unlike most types of mammalian cells, blastomeres do not require extracellular signals to survive in culture: when cultured from 1-cell up to 16-cell mouse embryos, they can survive and divide in the absence of exogenous proteins or signaling molecules, even when cultured as isolated single cells (Biggers *et al.*, 1971). Mouse blastomeres have an unusual resistance to experimentally induced apoptosis suggesting the likelihood that the apoptotic pathway is constitutively inhibited during early embryogenesis (Weil *et al.*,

1996). In the presence of CHX, blastomeres die with the characteristic features of apoptosis, suggesting that even blastomeres are capable of PCD and constitutively express the proteins required to execute the death program (Weil *et al.*, 1996). This result suggests that apoptosis is the default state for all cell types, even blastomeres.

Zebrafish blastomeres treated with inhibitors of transcription and DNA replication do not undergo apoptosis until mid-gastrulation. The effect of an inhibitor of translation on blastomere cell death has not been analyzed. Because the zebrafish early embryo relies heavily on maternally inherited factors, mRNA and protein, it is important to understand the significance of maternal control on cell survival at the translational level. The fact that the zebrafish early embryo does not have the capacity to execute transcription before the midblastula transition means that the early embryo relies on the proper execution of translation to regulate cell function. If zebrafish blastomeres require the synthesis of survival factors such as Bcl-2 to maintain the inhibition of proapoptotic cell death machinery, then treatment with cycloheximide should cause survival factor deprivation and in-turn allow the activation of programmed cell death.

Programmed Cell Death, Apoptosis, and Necrosis

Cell death plays a crucial role in the proper development of the vertebrate embryo (Glucksmann, 1951). Programmed cell death is a form of cell death that is activated by developmental or environmental stimuli and adapted for the well being of the organism (Lockshin and Williams, 1965; Lockshin, 1969). During development, this program may be activated for a number of reasons, which may include the elimination of unnecessary, irreparably damaged, or infected cells. In adult life, for every cell that is produced through mitosis, homeostasis of cell number requires that another cell be removed through programmed cell death.

Much of our current understanding of PCD stems from research done in the nematode *Caenorhabditis elegans*. During the development of the nematode, cell death occurs in a highly replicable fashion with 131 of 1090 somatic cells dying through PCD (Sulston *et al.*, 1983). Initial genetic screens done with mutants in which cell death was defective led to the identification of the *ced* (cell death defective) family of genes. These genes, *ced-3*, *ced-4*, and *ced-9*, are the central executioners of cell death in *C. elegans* (Yuan and Horvitz, 1990; Hengartner 1992; Yuan *et al.*, 1993; Xue *et al.*, 1996). Biochemical analyses of CED-3, CED-4, and CED-9 have allowed a more profound understanding of the mechanisms controlling these proteins in *C. elegans* (Wu *et al.*, 1997). Several

other caspases have also been found in *C. elegans* suggesting that the nematode contains more complex caspase cascades than previously thought (Shaham, 1998).

The caspase family of proteases plays a critical role in the proper execution of PCD during mammalian development and morphogenesis. Genetic studies done to identify mammalian homologues of the *ced* genes led to the identification of three structurally and functionally homologous genes, interleukin-1-beta converting enzyme (*ICE*), apoptosis protease-activating factor 1 (*Apaf-1*), and B cell lymphoma 2 (*Bcl-2*) (Miura *et al.*, 1993; Hengartner and Horvitz, 1994; Zou *et al.*, 1997). In *C. elegans*, CED-3 acts as the main effector protease. CED-3 is activated by the disruption of the CED-9/CED-4 complex by EGL-1 (del Peso *et al.*, 1998).

A similar effector protease regulatory system exists in mammals, which include Bcl-2, ICE, and Apaf-1. In addition, many cell death related genes have been cloned from the dipteran *Drosophila melanogaster* (White *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; Inohara *et al.*, 1997; Song *et al.*, 1997; Fraser *et al.*, 1997). In general, cell death programs are conserved throughout evolution from *C. elegans* to *H. sapiens*, but there are genes unique to invertebrates as well as genes unique to vertebrates. Furthermore, the single cell death genes of invertebrates have evolved to large families of similar genes in mammals.

In development, programmed cell death helps to shape the embryo in a highly guided and controlled fashion. The patterns of its occurrence led to the recognition that it was genetically controlled. There are two distinct modes of cell death that are predominant in vivo and in vitro. Apoptosis (controlled cell death) and necrosis (uncontrolled cell death) can be distinguished based on differences in morphological and molecular changes found in dying cells, and the two are considered to be unrelated.

Apoptosis is a term used to describe a form of programmed cell death observed in histological sections of mammalian tissues in which there was no other evidence of pathology such as an inflammatory response (Kerr *et al*., 1972). Apoptosis is characterized by cell shrinkage, condensation of the chromatin to the margins of the nuclear membrane, nuclear breakdown, membrane blebbing, and eventual fragmentation of the cell into apoptotic bodies that are engulfed *in vivo* by neighboring cells or phagocytes (as reviewed by Uren and Vaux, 1996). The apoptotic bodies are rapidly engulfed and degraded by neighboring cells, usually without disruption to the surrounding tissues. The fact that apoptotic cells or apoptotic bodies do not lyse *in vivo*, and thus do not contribute to inflammation, emphasizes the maintenance of cell membrane and apoptotic body integrity until late in the process. Necrosis on the contrary, occurs when a cell lyses following damage to a vital or many vital cellular processes.

Necrosis is characterized by cell swelling, formation of microvesicles and breakage of the plasma membrane, which allows leakage of the cytoplasm into the intercellular space (as reviewed by Uren and Vaux, 1996). In mammals, the necrotic cell debris is cleared by an inflammatory infiltrate of phagocytic cells, and tissue scarring is often associated with the process. Cells forced to undergo apoptosis in culture display all of the same characteristics up to membrane blebbing, but due to the absence of phagocytes in culture, cells may become necrotic.

In apoptosis morphological changes to the organelles are minor, except for some shrinkage, but with necrosis changes to the organelles, such as mitochondrial swelling, occur early. Necrotic pathophysiology involves a progression of translocase dysfunction in mitochondrial and plasma membranes, nonspecific increase of membrane permeability, and eventual physical disruption of membranes, all leading to many biochemical and structural abnormalities (Buja *et al.*, 1993).

The gross morphological changes seen during apoptosis occur relatively late in the death process. Many biochemically triggered events occur much earlier in the apoptotic process. One of the first signs of the beginning of apoptosis is the externalization of phosphatidylserine (PtdSer) from the inner leaflet of the cell membrane to the outer leaflet. PtdSer under normal

circumstances is present exclusively on the inner leaflet of the cell membrane. Exposure of PtdSer on the outer leaflet is believed to function as a signal for phagocytosis. There is recent evidence that not only is PtdSer externalized early in apoptosis, but that its synthesis is elevated in response to activation of the cell death pathway (Aussel *et al.*, 1998). This elevated synthesis is thought to occur in preparation for the apoptotic bodies that will eventually be formed. These latter also seem to require PtdSer for proper removal by phagocytes.

For the purpose of phagocytosis, macrophages of all types recognize PtdSer exposed on the surface of apoptotic lymphocytes (Krahling *et al.*, 1999). PtdSer exposure is also linked to the apoptosis related family of death proteases, the caspases. Sterol regulatory element binding-proteins become activated by caspase-mediated cleavage during apoptosis, and although a specific cleavage event has not been associated with PtdSer redistribution, caspase inhibitors have been shown to block it (as reviewed by Nicholson & Thornberry, 1997).

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments as well as single strand breaks ("nicks") in high molecular weight DNA. Biochemically, apoptosis is characterized by the degradation of chromatin, initially into large fragments of 50-300 kilobases and subsequently into smaller fragments that are monomers and oligomers of 150 base sequences, i.e., fragments of DNA are at the linker regions between

nucleosomes (Oberhammer *et al.*, 1993). Enzymatic labeling of free 3'-OH termini with modified nucleotides can be used to identify these DNA strand breaks. The TUNEL assay preferentially labels DNA strand breaks generated during apoptosis, allowing the discrimination of apoptosis from necrosis.

There is recent evidence that apoptosis and necrosis may have overlapping mechanisms controlling the decision to die (Leist *et al.*, 1997). During the development of the mouse embryo interdigital cells die in an attempt to form individual digits (Glucksmann, 1951). Caspase dependent apoptosis is responsible for the loss of these interdigital cells (Zakeri *et al.*, 1994). When caspase activation is inhibited by drugs or when mutant mice defective in cell death are analyzed, cell death still occurs in the interdigital webbing (Chautan *et al.*, 1999). This cell death was TUNEL negative and no evidence of chromatin condensation could be seen (Chautan *et al.*, 1999). One can infer from this evidence that there is a link between apoptosis and necrosis in some contexts.

Morphologically, irregular clumping of chromatin can be seen in the earlier stages of necrosis (Kerr *et al.*, 1995). As necrosis progresses to later stages of degeneration, the chromatin disappears and the nucleus may be discernible only as a faintly stained ghost (Kerr *et al.*, 1995). The cytoplasm of necrotic cells loses its basophilia and organized structure, and it becomes granular or finely vacuolated (Kerr *et al.*, 1995). The classic morphological

features seen during apoptosis, cell shrinkage, condensation of the chromatin to the margins of the nuclear membrane, nuclear breakdown, and membrane blebbing are not seen during necrosis.

The involvement of phosphorylation and gene expression in apoptosis implies that the process is energy dependent, an assumption that has been confirmed in several recent studies. Wherever ATP levels have been directly measured in apoptotic cells, no rapid drop has been observed (Ankarcrona *et al.*, 1995; Shimizu *et al.*, 1996a). Direct evidence demonstrating that ATP levels distinguish apoptosis from necrosis was illustrated by glutamate-induced neuronal cell death in which early necrosis was triggered by a rapid drop in mitochondrial $\Delta\psi_m$, and ATP depletion, but upon mitochondrial recovery ATP levels recovered, committing the neurons to apoptosis (Ankarcrona *et al.*, 1995).

In an attempt to elucidate how much ATP was required for apoptosis, Leist *et al.*, performed an ATP clamping experiment in which oligomycin treated cells were incubated in exogenous glucose, allowing the production of ATP by glycolysis (Leist *et al.*, 1997). Depletion of 50% of cellular ATP was sufficient to switch the mechanism of cell death from apoptosis to necrosis in staurosporine treated cells whereas a depletion of approximately 70% was required to switch the mode of cell death in Fas-triggered cells (Leist *et al.*, 1997).

Some of the best evidence for mechanistic overlap between apoptosis and necrosis comes from *in vitro* models of ischemia/reperfusion injury (Lemasters *et al.*, 1987). Hypoxia resulting from ischemia results in a rapid impairment of mitochondrial function due to inhibition of respiration, leading to a rapid and steep decline in $\Delta\psi_m$ and ATP (Lemasters *et al.*, 1987). These events can be mimicked in vitro by incubating cells in medium lacking glucose and all glycolytic energy sources plus an inhibitor of mitochondrial respiration, such as cyanide, rotenone, antimycin-A, or oligomycin. This chemical hypoxia ultimately results in necrotic cell death characterized by rapid and nearly complete ATP depletion, a drop in $\Delta\psi_m$, mitochondrial swelling, and loss of plasma membrane integrity (Lemasters *et al.*, 1987). Overexpression of bcl-2 can dramatically inhibit necrosis resulting from chemical hypoxia, supporting the belief that the two modes of cell death are under common mechanistic controls (Shimizu *et al.*, 1996a; 1996b). Caspase activation and DNA fragmentation were suppressed under conditions of ATP depletion (Eguchi *et al.*, 1997; Leist *et al.*, 1997). Thus the rate of ATP depletion seems to be an important factor in whether cells undergo an apoptotic or necrotic death.

Early Embryogenesis and Programmed Cell Death

A substantial number of cells of various types die during embryogenesis, allowing the formation of tissue structures in a highly controlled manner. During early embryogenesis, cell death apparently purges the embryo of damaged cells generated during cell proliferation and improper differentiation. During the first four cell divisions, normal cell death does not occur in blastomeres (Handyside and Hunter, 1986; Weil *et al.*, 1996). Cell death is first seen during blastocyst formation primarily in the inner cell mass in mouse and human blastocysts, and it appears that cell death in preimplantation embryos occurs as soon as the totipotent cells start to differentiate (Handyside and Hunter, 1986; Hardy *et al.*, 1989; Pierce *et al.*, 1989; Jurisicova *et al.*, 1995).

In humans, an oocyte is readily fertilized only for 24 hrs following ovulation, while insemination on the day following ovulation rarely results in a successful pregnancy (Wilcox *et al.*, 1995). Thus, it seems that an ovulated human egg ages rapidly, losing the ability to give rise to a viable conceptus within 24h of ovulation. In higher vertebrates if the ovum is not properly fertilized, death of the ovum eventually occurs. It has previously been demonstrated that unfertilized eggs and polar bodies are eliminated through apoptosis (Takase *et al.*, 1995; Warner *et al.*, 1998a). Thus, it seems that the

ovum is predestined to die unless fertilization and embryogenesis are successful.

The caspase family of death proteases has been implicated as the effectors of cell death in mouse preimplantation embryos (Exley *et al.*, 1999). All caspase mRNAs were detected in mouse oocytes while expression in preimplantation embryos varied in a stage-specific manner (Exley *et al.*, 1999). An assay for effector caspase enzymatic activity in mouse zygotes showed that even though transcripts for these caspases could not be detected in zygotes, proteolytic activity could be detected in polar bodies, fragmented zygotes, and zygotes treated with staurosporine (Exley *et al.*, 1999). This finding supports the view that the caspases are inherited as mRNA and synthesized as proenzymes during oogenesis, but the mRNA is degraded during zygote formation.

Invertebrates share many common pathways of apoptosis with vertebrates during early embryogenesis. Unfertilized starfish eggs arrest in postmeiotic interphase, and they eventually die if left unfertilized (Yuce and Sadler, 2001). Postmeiotic interphase arrested eggs persist for 16-20h, after which time the egg synchronously and rapidly die (Yuce and Sadler, 2001). The dying eggs extrude membrane blebs, fragment their cytoplasm, and condense their DNA (Yuce and Sadler, 2001). Activation of caspase-3 activity was detected in starfish eggs treated with the hormonal stimulant 1-methyladenine,

which causes maturation of starfish eggs (Sasaki and Chiba, 2001). Treatment of the eggs with the caspase-3-specific inhibitor Ac-DEVD-CHO, blocked membrane blebbing and fragmentation in eggs treated with 1-methyladenine (Sasaki and Chiba, 2001).

Blastomeres and preimplantation embryos are also susceptible to apoptosis (Warner *et al.*, 1998b). A few apoptotic cells can be observed in normal blastocysts, in what appears to be part of the normal developmental program (Hardy *et al.*, 1989; Jurisicova *et al.*, 1995). Blastomeres disassociated from 2- or 4-cell mouse embryos did not die when cultured for 18 or 24h with 1μM staurosporine, with or without cycloheximide. They remained phase-bright, had intact plasma membranes, normal looking nuclei that stained with bisbenzimide, and were TUNEL positive (Weil *et al.*, 1996). Blastomeres in intact 4-cell embryos behaved similarly, even with 10μM staurosporine, as did freshly ovulated mouse eggs (Weil *et al.*, 1996). Thus, it appears that blastomeres are unusually resistant to the lethal effects of staurosporine and cycloheximide, as all other normal, developing nucleated cell types tested undergo apoptosis with equivalent doses (Weil *et al.*, 1996).

When eggs or 1-4-cell embryos were treated with 10μM staurosporine, with or without cycloheximide, for 26-60 h, however, most of the cells died as judged by their abnormal morphology, condensed chromatin, and TUNEL

positive staining (Weil *et al.*, 1996). Thus, it seems that mouse blastomeres and eggs though resistant, are capable of undergoing apoptosis and do not need to initiate *de novo* protein synthesis to do so. By the 32-cell stage, mouse blastomeres develop into two distinct cell types, the inner cell mass and trophectoderm cells. When 32-64-cell blastocysts were treated with 1mM staurosporine and 10mg/ml cycloheximide for 18h, more than 90% of the cells died with the characteristic morphological features of apoptosis, indicating that both cell types are more sensitive than blastomeres to these drugs (Weil *et al.*, 1996). When inner cell mass cells were isolated and treated in the same manner, all of the cells died through classical apoptosis (Weil *et al.*, 1996). Thus, it seems that blastomeres become more sensitive to staurosporine and cycloheximide-induced apoptosis after they differentiate into their respective cell types.

Embryo fragmentation, the formation of anucleate cytoplasmic fragments, is a common feature seen during early human embryonic development (Jurisicova *et al.*, 1996). Programmed cell death is triggered in arrested fragmented human embryos (Jurisicova *et al.*, 1996). Human embryos examined 24h or more after cell cycle arrest often show characteristic features of apoptosis, including cytoplasmic, and nuclear changes, and DNA fragmentation. There is no known correlation between apoptosis and cytoplasmic fragmentation, but fragmentation might actually induce apoptosis

(Antczak and van Blerkom, 1999). Secondary necrosis is the ultimate fate of persistent cytoplasmic fragments that are not cleared by phagocytosis.

Deprivation of growth factors appears to be one of the pathways involved in the initiation of apoptosis in many developmental systems (Raff, 1992; Brison and Schultz, 1997). The levels of apoptotic death within blastocysts are regulated by survival factors produced both by the embryo and by its maternal component (Kane *et al.*, 1997; Brison and Schultz, 1997). During early rodent embryo implantation, apoptosis also contributes to the formation of the amniotic cavity (Coucouvanis and Martin, 1995).

Zebrafish Programmed Cell Death and Apoptosis

The zebrafish (*Danio rerio*) has arrived in the pantheon of genetic model organisms (Fishman, 1999). *D. rerio* is the fourth vertebrate to have its complete genome mapped, behind the maps of *H. sapiens*, *M. musculus*, and *R. norvegicus*. This accomplishment has made the zebrafish a crucial model in bridging the genetics of the invertebrates, *C. elegans*, and *D. melanogaster* and the higher vertebrates, *M. musculus*, and *H. sapiens*. The zebrafish embryo is an exceptional model for studying the interplay between genetic and environmental influences on apoptosis. Numerous mutant zebrafish lines demonstrating abnormal apoptosis have been identified (Nusslein-Volhard, 1994; Driever *et al*., 1994; Furuntani-Seiki *et al*., 1996; Haffter *et al*., 1996).

Large numbers of apoptotic early diplotene oocytes were observed during the transition from ovary-like undifferentiated gonadal tissue to testes during sex differentiation in presumptive male zebrafish (Uchida *et al*., 2002). In genetic all-females, apoptosis in a proportion of early diplotene oocytes was found in the undifferentiated gonads at 15-19 days post-hatching, probably as a result of programmed oocyte loss during ovarian development (Uchida *et al*., 2002). These findings support that oocyte apoptosis is the mechanism of testicular and ovarian differentiation in zebrafish.

There are three mitotic domains in the zebrafish early embryo (Kane *et al.*, 1992). Two are of blastoderm cells, the outer enveloping monolayered epithelial layer, and the inner, or "deep" mass of spherical cells and the third is the yolk syncytial layer (Kane *et al.*, 1992). Shortly after the emergence of these mitotic domains, individual deep cells leave the cell cycle as they become committed to their fates (Kimmel *et al.*, 1994). These terminal birthday divisions begin during midgastrulation at 8 to 10 hours post-fertilization (hpf) (Kimmel *et al.*, 1994).

Naturally occurring programmed cell death can be found in the zebrafish embryo in the tailbud and 1-somite stage, and during most of the stages following gastrulation (Yabu *et al.*, 2001a; Cole and Ross, 2001). Apoptotic cells were identified in the nervous system, associated sensory organs, retina, lens, cornea, otic vesicle, lateral line organs, and Rohon-Beard neurons (Cole and Ross, 2001). Apoptotic cells were also found in many non-neuronal structures such as the notochord, somites, muscle, tailbud, and fins (Cole and Ross, 2001).

Large numbers of apoptotic cells that were seen in 20 hpf zebrafish embryos were no longer visible in 22 hpf embryos (Cole and Ross, 2001). Zebrafish embryo tailbud cells that were identified as apoptotic 18-24 hpf were completely eliminated within 2 hrs (Cole and Ross, 2001). The fact that the zebrafish embryo contains an apoptotic program that is capable of such rapid

cell clearance suggests that the embryo must also have a highly evolved and efficient mechanism for apoptotic cell clearance.

The identification of a cornucopia of zebrafish genes with homology to mammalian apoptosis regulators has added the teleost to the list of organisms with highly evolved cell death pathways. The tumor suppressor p53 was cloned from zebrafish, and the transcripts were detected early during the formation of the zygote (Cheng *et al.*, 1997). Northern analysis demonstrated the transcript to be present and most abundant in zygotes and early-cleavage embryos less than 1 hpf, thereafter declining to barely detectable levels at 48 hpf (Cheng *et al.*, 1997). A similar temporal expression was detected for zebrafish L-myc, known to be present in maternally derived RNA, whereas zebrafish N-myc and the zebrafish homologue of the murine T gene were not detectable prior to the onset of zygotic transcription (Cheng *et al.*, 1997).

In addition, the cell surface death receptor TNF, of zebrafish ovary origin, and the apoptosis inducing ligand TRAIL were cloned from zebrafish (Bobe and Goetz, 2001). In zebrafish, the TRAIL-like cDNA hybridized with a 6-kb transcript present in most zebrafish tissues (Bobe and Goetz, 2001). This finding is in agreement with the constitutive expression of TRAIL mRNA in many mammalian tissues (Ashkenazi and Dixit, 1999). The TRAIL-like transcript was also detected in the ovary at all stages of the zebrafish ovarian cycle (Bobe and Goetz, 2001). On Northern blots, the ovarian TNF transcript

was predominantly expressed in the ovary and was detected in all the stages of the ovarian cycle (Bobe and Goetz, 2001). The constitutive presence of TRAIL and TNF transcripts suggests that both could play a role in the regulation of ovarian functions in zebrafish.

A homologue of the human apoptosis repressor Bcl-XL, Bcl-XL-like protein 1 (BLP1), was cloned and characterized from zebrafish (Chen *et al.*, 2001). BLP1 was expressed extensively during zebrafish embryonic development, and its detection in unfertilized eggs suggested a maternal origin (Chen *et al.*, 2001). BLP1 transcripts gradually decreased over time through the gastrula stage (Chen *et al.*, 2001). BLP1 mRNA levels increased again at the early somite stage, and then seemed to remain stable through hatching stage (Chen *et al.*, 2001).

A list of 37 other zebrafish genes with significant homology to mammalian apoptosis genes were identified from EST databases (Inohara and Nunez, 2000). Their analysis revealed a remarkable conservation of apoptosis pathways between zebrafish and mammals. The mitochondria mediate an initiation of the apoptosis pathway involving many of the Apaf-1 related components that were present in zebrafish (Inohara and Nunez, 2000). Several zebrafish proteins with significant homology to mammalian caspases including Caspase-2, -3, -6, -8 ,-9, and -13 were also identified (Inohara and Nunez, 2000).

Caspase-3, the major effector caspase involved in apoptosis, was cloned and characterized from zebrafish embryos (Yabu *et al.*, 2001b). Caspase-3 mRNA was detected as early as the formation of the zygote (Yabu *et al.*, 2001b). Northern blot analysis revealed that caspase-3 mRNA was present in all developmental stages in the zebrafish embryo (Yabu *et al.*, 2001b). Because early embryos at the 4-cell and 1k-cell stages had high levels of capase-3 mRNA, it was thought to have been inherited as a maternal factor (Yabu *et al.*, 2001b). In addition, caspase-3 was expressed after gastrulation by zygotic genome activation, as were other housekeeping genes (Yabu *et al.*, 2001b). Overexpression of zebrafish caspase-3 induced apoptosis and increased ceramide levels in zebrafish embryos (Yabu *et al.*, 2001b).

Normally developing embryos have only low levels of caspase-3 activity (Yabu *et al.*, 2001a). When zebrafish embryos were treated by heat shock, caspase-3 increased markedly after 6 h recovery at 28.5^{o}C (Yabu *et al.*, 2001a). In 3 hpf embryos treated with UV irradiation at 20 mJ/cm^{2}, caspase-3 activity increased beginning at 8 hrs post-irradiation, with the activity eventually increasing by 7.5 times that of normal embryos after 12 hrs (Yabu *et al.*, 2001a).

No apparent TUNEL staining is observed at early embryonic stages, such as the 1000-cell, sphere, 50% epiboly and shield stages (Yabu *et al.*, 2001a). Several TUNEL-positive cells were observed during the tailbud and 1-somite stages in normal embryos (Yabu *et al.*, 2001a). This finding supports the belief

that naturally occurring programmed cell death does not occur until zygotic control of the embryo is achieved. Severe stresses, such as heat shock and irradiation, produced strong TUNEL-positive embryos (Yabu *et al.*, 2001a). When 3 hpf embryos were treated by heat shock and irradiation, TUNEL-positive cells were observed 8 hrs post-treatment (Yabu *et al.*, 2001a).

In 1997, Ikegami *et al.* first observed that the zebrafish embryo had no capability to undergo checkpoint induced arrest of cell proliferation until the midblastula transition (MBT), which begins at ~3 hpf (Ikegami *et al.*, 1997a, b). The zebrafish MBT is characterized by lengthening of the cell cycle, loss of cell synchrony, activation of transcription, and appearance of cell motility (Kane *et al.*, 1993). Superceding a 15-minute oscillator that controls the first nine cell cycles, the nucleocytoplasmic ratio appears to govern the MBT (Kane *et al.*, 1993).

During the several cycles after the MBT begins, cycle length is correlated with the reciprocal of the blastomere volume, suggesting a continuation of cell cycle regulation by the nucleocytoplasmic ratio during an interval termed the MBT period (Kane and Kimmel, 1993). As the MBT begins, the embryo simultaneously enters a maternal-to-zygotic transition (MZT), which marks the point that the zebrafish embryo begins to acquire the capability to undergo checkpoint-induced apoptosis (Ikegami *et al.*, 1997a; 1997b).

The MZT is characterized by depletion or degradation of maternal determinants (RNAs and proteins) and their replacement by zygotic RNA and proteins through zygotic transcription (Kane *et al.*, 1996). It is hypothesized that the embryo acquires a general capability for apoptosis as a consequence of passage through the MZT (Ikegami *et al.*, 1999). Gastrula stage, 6-10 hpf, defines a critical phase in zebrafish development with respect to the response to camptothecin (Ikegami *et al.*, 1997a; Ikegami *et al.*, 1999). In response to an early, pre-gastrulation, treatment with camptothecin, apoptosis was induced at a time corresponding approximately to mid-gastrula stage in control embryos, but not before this time (Ikegami *et al.*, 1997a; Ikegami *et al.*, 1999).

The same apoptotic response to blockage of DNA replication can also be induced by early, pre-MBT, treatment with the DNA synthesis inhibitors hydroxyurea and aphidicolin (Ikegami *et al.*, 1999). Chronic treatment of 4 and 8-cell zebrafish embryos with aphidicolin and hydroxyurea caused the death of the embryos at 8-10 h of age (Ikegami *et al.*, 1999). If the same treatments were applied to embryos between the 16 cell stage and the MBT, most of the embryos survived past gastrulation stage in control embryos (Ikegami *et al.*, 1999). In contrast, treatment of post-MBT embryos with aphidicolin failed to produce cell-proliferation arrest, apoptosis, or embryo death (Ikegami *et al.*, 1999).

Treatment of post-MBT embryos with hydroxyurea produced a high number of unusual nuclei, and some apoptotic cells, yet most of the cells continued to proliferate and the embryos remained alive (Ikegami *et al.*, 1999). In response to all the treatments, the enveloping and deep cell layers in early cleavage stage embryos undergo cell cycle arrest (Ikegami *et al.*, 1999). Fragmentation of nuclei can be blocked by the caspase-1,4,5 inhibitor Ac-YVAD-CHO, but not by the caspase-2,3,7 inhibitor Ac-DEVD-CHO (Ikegami *et al.*, 1999). Treatment of 9 hpf zebrafish embryos with camptothecin (a topoisomerase I inhibitor) causes cells to become apoptotic within 20 minutes (Ikegami *et al.*, 1999). This rapid activation of apoptosis has led researchers to hypothesize that the activation of apoptosis in zebrafish embryos does not require mRNA transcription (Ikegami *et al.*, 1999).

Pre-MBT treatment with the microtubule destabilizer nocodazole produces complete destruction of all nuclei in the deep cell layer of zebrafish embryos (Ikegami *et al.*, 1997b). Nocodazole-induced apoptosis in the deep cell layer can be blocked by the caspase-1, 4, 5 inhibitors Ac-YVAD-CHO and Ac-YVAD-CMK (Ikegami *et al.*, 1997b).

The initial observation that synthetic caspase inhibitors were able to block cell death in the nocodazole treated embryos led Ikegami's group to hypothesize that a homologue of the C. elegans ced-9, 4, 3 pathway is involved

in control over apoptosis in the developing zebrafish embryo (Ikegami *et al.*, 1997b).

Xenopus Programmed Cell Death and Apoptosis

The South African clawed toad, *Xenopus laevis*, shares many common developmental themes with the zebrafish during early embryogenesis, including the presence of a maternally encoded cell death program. Two distinct cell death programs have been identified in the *Xenopus* during the time between fertilization and the tadpole stage (Anderson *et al.*, 1997; Hensey and Gautier, 1997; 1998). During *Xenopus* early embryogenesis, the midblastula transition functions as a developmental milestone in which a maternally regulated checkpoint turns on at the beginning of the transition, purging the embryo of damaged cells (Anderson *et al.*, 1997). This event was found to be a maternally encoded cell death program that is activated at the onset of gastrulation following damage to the pre-midblastula transition embryo, resulting in the death of non-viable cells (Anderson *et al.*, 1997; Hensey and Gautier, 1997).

This maternal cell death program is activated under tightly regulated developmental control, occurring only at the single cell level. The maternally encoded cell death program is under the control of a developmental timer that is turned on upon fertilization. The contents of this cell death program are inherited as maternal factors (mRNA and protein), by the egg prior to fertilization (Hensey and Gautier, 1997). This cell death program has been activated by irradiation induced DNA damage, and inhibitors of transcription,

translation, and replication, in embryos between the time of fertilization and the midblastula transition. The activation of this program does not depend on the type of stress applied, cell cycle progression or *de novo* protein synthesis. If the same treatments are applied to the *Xenopus* embryo after the midblastula transition, the apoptotic response is not seen (Hensey and Gautier, 1997).

Treatment of 2-cell embryos with α-amanitin, an inhibitor of transcription, had no effect on the early cell divisions, whereas treatment with the protein synthesis inhibitor cycloheximide caused an arrest in cell division within 1 h of treatment, due to the inhibition of cyclin synthesis (Hensey and Gautier, 1997). When the 4-cell arrested embryo was exposed to cycloheximide for 7.5 hrs, equivalent to stage 9 in untreated controls, TUNEL staining was still not detected (Hensey and Gautier, 1997). When cycloheximide was applied continuously at concentrations that block 97% of protein synthesis, *Xenopus* embryos arrested cell division but did not die until a time when they would normally be gastrulating, approximately stage 10.5, which is the time when they become TUNEL positive (Hensey and Gautier, 1997).

A typical characteristic apoptotic morphology was seen in *Xenopus* embryos exposed to cycloheximide, which included the budding of the nucleus to produce discrete nuclear fragments of varying size and chromatin content,

which were still surrounded by a double membrane (Hensey and Gautier, 1997). Cycloheximide pulsed embryos examined prior to the midblastula transition contained chromatin that appeared damaged, without apoptotic bodies, suggesting that apoptosis did not occur prior to the midblastula transition (Sible *et al.*, 1997). The use of emetine, another protein synthesis inhibitor, confirmed the results obtained with cycloheximide, that cycloheximide induced apoptosis resulted from protein synthesis inhibition, and not from a particular toxicity of the drug (Sible *et al.*, 1997).

In *Xenopus*, the duration of the cycloheximide treatment is not what determines the time of death, as embryos exposed for 6, 8, and 10hrs, all underwent cycloheximide-induced cell cycle arrest, and all died synchronously at a time equivalent to stage 10.5 (Hensey and Gautier, 1997). The apoptotic response was never elicited prior to stage 10.5. This result could be due to the embryo's inability to activate the apoptotic pathway prior to stage 10.5, or to an excess of an inhibitory component of the cell death pathway present in *Xenopus* embryos before stage 10.5.

This maternally inherited inhibitor could be degraded using a mechanism similar to that seen in cyclin degradation between the midblastula transition and gastrulation (Howe and Newport, 1996). Because all the apoptotic responses seen in *Xenopus* early embryos took place consistently at the early gastrula stage, it is reasonable to assume that there is some "time-switched" mechanism that

recognizes the severely damaged cellular state and activates the apoptotic program shortly after the midblastula transition.

The second cell death program found in the *Xenopus* is responsible for naturally occurring cell death, or programmed cell death, that occurs as a part of normal *Xenopus* embryonic development (Hensey and Gautier, 1998). Programmed cell deaths begin at the onset of gastrulation and persist through the tadpole stage (Hensey and Gautier, 1998). The first naturally occurring programmed cell deaths that occur in the early *Xenopus* embryo commence between stages 10.5 and 11.5 of development (Hensey and Gautier, 1998). The earliest TUNEL-positive embryos were detected at stage 10.5, the beginning of gastrulation (Hensey and Gautier, 1998).

Cell-free systems have been developed to study apoptosis in *Xenopus* using cytoplasmic extracts derived from eggs (Newmeyer *et al.*, 1994). The cell-free systems described mimicked apoptosis in several aspects. Chromatin condensation, the formation of a ladder of oligonucleosomal DNA fragments, and caspase activation were all evident in the system (Newmeyer *et al.*, 1994; Kluck *et al.*, 1997). The *Xenopus* cell-free system was the first used to highlight the requirement for mitochondria in apoptosis (Newmeyer *et al.*, 1994). The importance of cytochrome-c in apoptosis was identified through the use of the cell-free system (Kluck *et al.*, 1997). The fact that the unfertilized *Xenopus* egg

contains such an elaborate cell death pathway supports the argument that the cell death machinery found in the early embryo is of maternal origin.

It is likely that the apoptosis machinery present in *Xenopus* egg extracts functions *in vivo*, in the case of oocyte atresia, a form of apoptosis (Smith *et al.*, 1991). Atresia in rat ovarian granulosa cells *in vivo* is accompanied by the morphological changes characteristic of apoptosis and internucleosomal DNA fragmentation, an indicator of active DNA degradation (Hughes and Gorospe, 1991; Tilly *et al.*, 1992). The fact that naturally occurring and induced cell death are never detected before gastrulation shows that maternal factors are highly influential in the regulation of *Xenopus* embryonic apoptosis.

Cycloheximide Induced Apoptosis

Cycloheximide (CHX) is a glutarimide antibiotic derived from a microbial source that inhibits eukaryotic, but not prokaryotic protein synthesis (Wilkie and Lee, 1965; Jackson and Studzinski, 1968). Protein synthesis is inhibited in eukaryotic organisms by the interaction of cycloheximide with the translocase enzyme, inhibiting peptidyl transferase activity. At the cellular level, cycloheximide blocks the translation of messenger RNA on cytosolic, 80S and 60S ribosomes, but does not inhibit organelle protein synthesis (Obrig *et al.*, 1971; Setkov *et al.*, 1992; Suzuki *et al.*, 1992).

This specific biochemical inhibition makes cycloheximide a powerful tool in the study of many cellular processes. Although macromolecular synthesis was initially considered to be required for active cell death, it now appears that, depending on the experimental system used, the presence of protein synthesis inhibitors can lead either to prevention or stimulation of apoptosis (Wyllie *et al.*, 1984; Ucker *et al.*, 1989; Oppenheim *et al.*, 1990; Whyte *et al.*, 1991; Ning *et al.*, 1995). In comparative studies done with cells pretreated with cycloheximide (1-100 μg/ml) and cells not treated with the inhibitor, researchers have helped determine the role of protein synthesis in apoptosis (Pober *et al.*, 1998; Satoh *et al.*, 1998; Snider *et al.*, 1999).

Using several lines of evidence, researchers have shown that the cell death induced by CHX is bona fide apoptosis (Blom *et al.*, 1999; Lemaire *et al.*, 1999; Tang *et al.*, 1999). Induction of apoptosis by cycloheximide *in vivo* and *in vitro* is usually interpreted as the loss of an inhibitor or inhibitors of apoptosis (Martin *et al.*, 1990; Collins *et al.*, 1991; Ledda-Columbano *et al.*, 1992). Cycloheximide can trigger apoptosis alone or in conjunction with other agents (e.g., tumor necrosis factor-α) (Tsuchida *et al.*, 1995). Cycloheximide has been shown to exert two opposite effects in B lymphocytes in a dose dependent manner (Lemaire *et al.*, 1999). High doses of cycloheximide (2.5 μg/ml) inhibited protein synthesis (> 90%) and greatly increased B cell apoptosis (Lemaire *et al.*, 1999).

Low doses of cycloheximide (0.05 μg/ml) modestly inhibited protein synthesis (< 15%) and reduced spontaneous as well as drug-induced apoptosis (Lemaire *et al.*, 1999). It was initially stated that B lymphocyte apoptosis does not require *de novo* protein synthesis because spontaneous apoptosis was increased by cycloheximide (Illera *et al.*, 1993; Norvell *et al.*, 1995). This result favored the hypothesis of the pre-existence of a death machinery that must be restrained by the continuous synthesis of short-lived protective proteins, including members of the Bcl-2 family (Illera *et al.*, 1993).

A large body of evidence has shown that cycloheximide can potentiate, and in some cases (e.g., TNF-α stimulation and staurosporine) may be

necessary for the apoptogenic effects of certain death stimuli (Tsuchida *et al.*, 1995; Jacobson *et al.*, 1996). Jurkat cells exposed to 20μg/ml cycloheximide became apoptotic within 4-6 hrs, evidenced by PARP cleavage and annexin V staining of cell-surface phosphatidylserine. Fas ligand was not expressed in Jurkat cells exposed to cycloheximide before or after exposure, suggesting that the activation of apoptosis did not involve the Fas receptor. Expression of a Fas-associated death domain dominant negative (FADD-DN) effectively inhibited cycloheximide induced apoptosis, suggesting that a FADD-dependent mechanism functioning in a receptor-independent manner was primarily involved. Immunoblotting revealed the processing and activation of caspase-8 during cycloheximide-induced Jurkat cell death (Tang *et al.*, 1999).

In addition, others demonstrated that FADD and caspase-8 coalesce into what appeared to be perinuclear death effector filaments (DEFs) (Perez and White, 1998; Siegel *et al.*, 1998) in both wild type and FADD-DN Jurkat cells treated with cycloheximide (Tang *et al.*, 1999). Surprisingly, FADD-DN expression in Jurkat cells before cycloheximide exposure, effectively inhibited caspase-3 activation induced apoptosis (Tang *et al.*, 1999). Bone marrow neutrophils isolated from caspase-3 KO mice were resistant to cycloheximide induced apoptosis upon exposure, supporting the Jurkat cell data, and supporting the hypothesis that caspase-3 is most likely required for cycloheximide induced cell death (Woo *et al.*, 1998).

Microinjection of cycloheximide into cleavage stage zebrafish embryos caused a reduction in the levels of proliferating cell nuclear antigen (PCNA) after 10 to 25 minutes (Yarden and Geiger, 1996). The reduction in PCNA levels demonstrates the effectiveness of cycloheximide at inhibiting protein synthesis in zebrafish embryos. The use of cycloheximide should shed light on the importance of protein synthesis in maintaining the levels of survival factors and inhibition of apoptosis before the zebrafish MZT/MBT. Given the extensive use of cycloheximide in such a variety of model systems of apoptosis, its use in zebrafish early embryos should add to the understanding of cycloheximide-induced death during embryogenesis, and the role survival factors play during this period.

The Caspases and Apoptosis

Caspases are cysteinyl proteases that cleave defined tetrapeptide sequences terminating in aspartic acid residues. They are responsible for the orderly dismantling of cellular substrates during PCD. The identification of the first caspase (caspase-1 or ICE) came with the realization that a mature caspase is generated by proteolytic processing at critical aspartic acids and hence a mature caspase may activate its precursor as well as other procaspases (Thornberry *et al.*, 1992).

Knowledge of the large family of related cysteine proteases has steadily increased over the past nine years, to the point where a unified nomenclature had to be created just to be able to keep track of them. Several lines of evidence indicate that this family, the cysteinyl aspartate-specific proteases (caspases), are involved in many types of cell death. Caspases, one of many types of enzymes that participate in apoptosis, are required for the accurate and limited proteolytic cleavage of many cytosolic and cytoskeletal proteins. Although 14 caspases are known in mammals, only a few have been described in other organisms.

The caspases are first synthesized as pro-enzymes and are present throughout the cell as inactive proteases. There are two main types of caspases; the first are the initiator caspases, which interact with adapter molecules that

associate with either cell surface receptors or cytosolic receptors throughout the cell. The second group of caspases is the effector caspases; these are the workhorses of apoptosis. Once activated by the initiator caspases, they become autocatalytic, cleaving both their own proenzymes and the substrates with exposed cleavage sites. Caspases are not the only enzymes that participate in apoptosis, because nucleases and protein kinases may also participate, but they are absolutely required for the accurate and limited proteolytic events that are manifested in this type of programmed cell death.

Although the morphological changes that occur in cells undergoing apoptosis are striking, there is no randomly occurring proteolysis of cellular constituents, as might be suggested by the gross ultrastructural changes. In reality, the caspases are specifically designed for proteolytic cleavage of many key constituents. The substrates cleaved include a wide range of proteins, including DNA repair enzymes, DNA replication proteins, cell-cycle regulators, cell adhesion proteins, nuclear structure proteins, cytoskeletal proteins, and nuclear envelope proteins. Otherwise, caspase cleavage sites are rare in proteins. Such proteolysis is directly responsible for the characteristic morphological changes associated with apoptosis and, ultimately, for cell death. The list of substrates for the caspases is growing steadily as we evolve a better understanding of what the caspases are capable of cleaving.

The inactive state of pro-caspase-3 is tightly regulated under normal conditions. Under conditions conducive to apoptosis, the zymogen is activated and the proteolytically active form is assumed. Caspase-3 expression is conserved and its role in the development of the embryonic neuronal system has been established. An array of abnormalities exhibited in the nervous system of caspase-3 knockout mice was due to reduced brain cell apoptosis, suggesting a major role for caspase-3 in neuronal death (Kuida *et al.*, 1996). Caspase-3 also plays a role in stress induced apoptosis as it was shown that caspase-3 was activated in stress-induced apoptosis in zebrafish embryos (Yabu *et al.*, 2001b).

The active form of caspase-3 is primarily found in the cytosol, yet it may translocate to organelles, i.e., the nucleus where it endoproteolytically cleaves proteins, such as PARP, that contain a Asp-Glu-Val-Asp (DEVD) motif (Zhivotovsky *et al.*, 1999). Once the caspases are activated, several important proteins, including fodrin, β-actin, $DNApk_{cs}$, and lamin B, are also cleaved. The cleavage of these proteins allows the display of a characteristic morphology seen in apoptotic mammalian cells, including PtdSer translocation, chromatin condensation, nuclear fragmentation, and membrane blebbing. The activation of the caspases appears to be essential for the execution of classical apoptosis (Yuan *et al.*, 1993).

The selective localization of pro-caspases in different subcellular compartments may play an important, but yet unknown role in their activation. The localization of pro-caspase-3 is strictly controlled during normal conditions, in which the zymogen can be found only in the cytosol, mitochondria, and microsomes. The translocation of active caspases to other subcellular compartments appears to be critical for the development of apoptosis (as noted by Zhivotovsky *et al.*, 1999). Under conditions conducive to apoptosis, the zymogen is activated and the active form can then be found in the nucleus where it can do damage to caspase-3 nuclear substrates (Zhivotovsky *et al.*, 1999). Caspase recruitment and oligomerization mediated by adaptor molecules constitutes a basic mechanism of caspase activation, and this basic mechanism is what makes the caspases such efficient protein killers. To add to this efficiency, cells have evolved a means to ensure that if one caspase pathway fails, others can be activated.

The importance of the caspases in the development of classical apoptosis can be seen in cells that are forced to over-express Bax, a known cell death promoter (Xiang *et al.*, 1996). These same cells were treated with the cell-permeable caspase inhibitor Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD-FMK), and cell death was partially delayed. Although caspase activity was completely inhibited, they still died. Yet they did not die with classical apoptotic morphology; the morphology was more representative of a necrotic

death. Treatment of B lymphocytes with Z-Asp-Glu-Val-Asp-chloromethylketone (Z-DEVD-CMK) and Z-VAD-FMK totally prevented drug induced caspase activation and apoptosis but it did not prevent the death of the cells (Lemaire *et al.*, 1998). These findings support the argument that apoptosis and necrosis may share a common initiation pathway, the final issue being determined by the presence of an active caspase and sufficient energy to run the machinery.

The only endogenous caspase inhibitor to date is the X-linked IAP (XIAP) protein, which has been shown to inhibit caspases-3, and 7. As a major form of posttranslational modification, phosphorylation is another method employed to modulate caspase activity. One example is the phosphorylation of caspase-9 by Akt (Cardone *et al.*, 1998). Akt, a serine-threonine protein kinase downstream of phosphatidylinositol 3-kinase, is implicated in apoptosis suppression mediated by growth factor receptors. Phosphorylation of caspase-9 by Akt inhibits caspase activity in vitro and activation in vivo. Not surprisingly, inhibition of caspases is a strategy adopted by viruses in their attempt to elude the response of apoptosis to the infiltration of the virus into the cell. Many viruses penetrate cells, highjack the cell's protein synthesis mechanisms, and begin to produce viral inhibitors of cell death.

Poly (ADP-ribose) polymerase (PARP), a major caspase substrate, is a nuclear zinc finger DNA-binding protein that detects and binds to DNA strand

breaks. At a site of DNA breakage, PARP catalyzes the transfer of the ADP-ribose moiety from its substrate, NAD^+, to a limited number of protein acceptors involved in chromatin architecture or in DNA metabolism. Apoptotic cells in PARP mutants are morphologically abnormal in that they show retarded cell shrinkage and nuclear condensation. These mutants are also more sensitive to apoptosis induced by DNA alkylation, and rescue of these mutants with functional PARP restores the viability of the mutants (Oliver *et al.*, 1998). During apoptosis in human cells, the 116kDa PARP is cleaved into two fragments of relative molecular mass 85 and 27 kDa.

The insertion of an uncleavable PARP (lacking the caspase-3 cleavage site) into mouse embryonic fibroblast (MEF) cells causes a delay in cytoplasmic blebbing and nuclear disassembly during apoptosis (Oliver *et al.*, 1998). This outcome shows that PARP not only slows down nuclear apoptosis, but it also interferes with the initial cytoplasmic events leading to cell membrane blebbing. It has also been shown that UV-irradiation increases poly(ADP-ribosyl)ation of nuclear proteins, particularly of histone H1 (Yoon *et al.*, 1996). A PARP inhibitor, 3-aminobenzamide, prevented both internucleosomal DNA fragmentation and histone H1 poly(ADP-ribosyl)ation in cells treated with UV (Yoon *et al.*, 1996). Yoon *et al* concluded that poly(ADP-ribosyl)ation of histone H1 protein in the early stage of apoptosis facilitates internucleosomal DNA

fragmentation by increasing the susceptibility of chromatin to cellular endonuclease.

Mitochondria and Apoptosis

For some time it was thought that mitochondria played no role in active cell death, but now it appears that mitochondria are central executioners in apoptosis. In *C. elegans* the discovery of CED-9 and its essential function in cell death led to the realization that it had a human homologue bcl-2. The oncoprotein bcl-2 was the first discovered mammalian cell death regulator. Bcl-2 is a membrane-associated protein capable of preventing cell death in several cell death cascades. Bcl-2 is located predominantly in the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane. This membrane association is of functional significance as mutant bcl-2 molecules lacking this membrane anchorage capacity are often less effective at preventing apoptosis (Schendel *et al.*, 1997). Bcl-2 has the ability to bind inhibitors of its own function, including members of the bcl-2 subfamily. Bcl-2 activity is associated with multiple means of initiating and effecting cell death, such as those involving growth factor deprivation, irradiation, hormones, oxygen radicals, and cytochrome-c leakage (Zhong *et al.*, 1993).

Mitochondria contribute to apoptosis signaling via the production of reactive oxygen species. The means by which bcl-2 protects against reactive oxygen species remains unclear. In some systems, bcl-2 appears to influence the generation of oxygen free radicals (Kane *et al.*, 1993) whereas in other cases

it does not affect reactive oxygen species production but does prevent oxidative damage to cellular constituents (Hockenbery *et al.*, 1993). It has also been proposed that it functions as a pro-oxidant and influences the levels of reactive oxygen species, in this way inducing endogenous cellular antioxidants (Steinman, 1995). The bcl-2/reactive oxygen species relationship remains poorly understood.

Bcl-2 deficient mice are characterized by massive apoptotic involution of the thymus and spleen, hypopigmented hair, and polycystic kidney disease (Veis *et. al.*, 1993). However, many regions that would normally show signs of bcl-2 expression in normal mice show normal morphology even when bcl-2 has been knocked out (Veis *et. al.*, 1993). One hypothesis is that regions that would normally show low to moderate bcl-2 expression during development have homologous genes from the bcl-2 subfamily that take over its function in knockout mice. This replacement gene product would allow normal development in as many organ systems as possible in the absence of bcl-2 expression.

The recent identification of genes structurally related to the bcl-2 gene, such as bcl-xs, bcl-xl, bax, and mcl-1, indicates an additional level of complexity in the control of cell death. Apoptosis protease-activating factor 1 (Apaf-1) has been found to be the link between bcl-2 and the proteases forming a three component complex. Bcl-2 can also act by regulating the release from

mitochondria of apoptosis inducing factor (AIF). One means of studying the effects of bcl-2 is to use cell-free systems. Study of several systems has led to different suggestions for functions of bcl-2.

These cell-free systems have been developed based on *Xenopus* egg extracts in which recombinant bcl-2 was able to prevent protease activation and subsequent apoptotic effects. This system is based on cytosol derived from apoptotic cells, which have a heavy membrane fraction, enriched in mitochondria (Kluck *et al.*, 1997). Sf9 cell (human Bcl-2 protein baculovirus-encoded rat cells) lysates have also been used in a cell-free system to test the ability of bcl-2 to prevent nuclear disintegration. Recombinant human bcl-2 produced in baculovirus was added to the Sf9 apoptotic cell lysates to test its ability to protect nuclear morphology, and the lysates protected the nuclei from apoptosis (Newmeyer *et al.*, 1994).

Several changes in mitochondrial biogenesis and function are associated with the commitment to apoptosis. A fall in the transmembrane potential ($\Delta\psi_m$) occurs before the fragmentation of the DNA into oligonucleosomal fragments (Vayssiere *et al.,* 1994; Petit *et al.*, 1995). This drop in $\Delta\psi_m$ is responsible for a defect of maturation of mitochondrial proteins synthesized in the cytoplasm (Mignotte *et al.*, 1990), cessation of mitochondrial translation and an uncoupling of the oxidative phosphorylations (Vayssiere *et al.*, 1994). The

drop of $\Delta\psi_m$ has also been observed during apoptosis of thymocytes induced by dexamethasone (Petit *et al.*, 1995).

A drop of $\Delta\psi_m$ has also been seen during apoptosis of the neurons deprived of nerve growth factor (Marchetti *et al.*, 1996). Therefore, it seems that the drop of $\Delta\psi_m$, an event that can be slowed by cyclosporine A, is a universal characteristic that accompanies apoptosis, independently of the induction signal and of the cell type (Kroemer *et al.*, 1995). The permeability of mitochondria is regulated by pores in the inner membrane of mitochondria. The opening of the pores in a step function called permeability transition (PT), PT can be blocked by a very low concentration of cyclosporin A.

These pores, permeable to compounds of molecular mass up to 1500 daltons, are formed under specific conditions. The opening of these pores allows the equilibration of ions and respiratory substrate between cytosol and mitochondrial matrix, leading to the reduction of the membrane potential and the arrest of ATP synthesis (Petronilli *et al.*, 1994). Most researchers believe that the opening of the PT pores is involved in the disruption of $\Delta\psi_m$ observed during the effector phase of apoptosis.

Cytochrome c is an essential component of the mitochondrial respiratory chain: it accepts an electron from cytochrome-c reductase and passes it to cytochrome c oxidase. It is a soluble protein that is located in the

intermembrane space and is loosely attached to the surface of the inner mitochondrial membrane. Cytochrome c is translated on cytoplasmic ribosomes as apocytochrome c and follows a unique pathway into mitochondria that does not require the signal sequence, electrochemical potential and general protein translocation machinery (Mayer *et al.*, 1995).

The apoprotein, on entry into the intermembrane space, gains a heme group to become the fully folded holocytochrome c. This globular positively charged protein can no longer pass through the outer mitochondrial membrane and is thought to become electrostatically attached to the inner membrane. How cytochrome c is released from the inner membrane space to the cytosol is still under investigation. Nevertheless, the polypeptide chain and the heme prosthetic group of cytochrome c are required to activate the caspases. The shift of cytochrome c requires activated cytosolic factors and is blocked by overexpression of bcl-2. As previously discussed, the recently discovered pore-forming properties of some bcl-2 subfamily members, like Bax, has led to the proposition that these proteins might directly modulate the permeability of the outer mitochondrial membrane to cytochrome c (Schendel *et al.*, 1997).

Although apoptosis can occur in the absence of detectable cytochrome-c release, efflux of cytochrome-c from mitochondria appears to be a critical coordinating step in one of the major killing programs (Li *et al.*, 1997). Bcl-2 can also block the pore-forming properties of bax. These arguments are the

basis of the hypothesis that these proteins are constituents of the mitochondrial permeability transition pores. One interpretation is that bax promotes cell death by allowing the efflux of ions and small molecules across the mitochondrial membranes, thus triggering permeability transition, whereas bcl-2 counteracts this effect. Injected cytochrome c can make an otherwise normal cell undergo apoptosis (Zhivotovsky *et al.*, 1998). The source of cytochrome c must be from a higher eukaryote for apoptosis to be induced, as cytochrome c from the yeast *S. cerevisiae* did not induce apoptosis in wild type NRK cells (Zhivotosky *et al.*, 1998). The same results were shown with biotinylated cytochrome-c, which was considered to be an inactive form. Cells of many types that over-expressed bcl-2 were partially resistant to induction of killing by cytochrome c (Zhivotosky *et al.*, 1998). This experiment was repeated in many cell types with similar results.

The ability of Bcl-2 to control cytochrome c release and to regulate many other pro-apoptotic molecules is an important factor in cell homeostasis and survival. Conversely, the ability of bax to bind bcl-2 and inhibit its anti-apoptotic effects is critical to a cell scheduled for elimination in a non-inflammatory manner. The mechanisms involved in cytochrome c release and the ability of bcl-2 to control its presence in the cytosol needs further elucidation, as does the structure of the complex that forms between the two molecules.

Summary and Hypothesis

All embryos possess the ability to eliminate unnecessary and damaged cells through programmed cell death. A substantial number of cells of various types die during embryogenesis because of exogenous proapoptotic signaling, survival factor deprivation, or improper differentiation. Apoptosis is the predominant form of programmed cell death during embryogenesis.

The zebrafish early embryo regulates its ability to undergo natural and drug-induced apoptosis in a stage dependent manner. The zebrafish early embryo is incapable of apoptosis before mid-gastrulation, as determined by the application of drugs, before the midblastula transition, that inhibit DNA synthesis and transcription, and that destabilize microtubules. Once the treated embryos developed to a time equivalent to mid-gastrulation in control embryos, they all synchronously underwent apoptosis. Treatment with the same drugs after the midblastula transition either had no effect on the embryo or led to apoptosis.

The zebrafish embryo relies on the proteolytic activity of the caspase family of proteases to orchestrate the elimination of unwanted cells. The aim of this investigator is to elucidate the extent to which caspase-3 activation contributes to the morphology of zebrafish apoptosis and to elucidation the

function and biological origin of the cell death program found in the zebrafish early embryo.

CHAPTER II

Methods and Materials

Fish Stocks and Fish Aquaculture

Wild type zebrafish were used for all experiments. The staging of zebrafish embryonic age was done in accordance with an established protocol (Kimmel *et al.*, 1995). Aquaculture methods were adapted from an established protocol (Westerfield, 1995). Zebrafish adults are maintained as schools of 30 fish in 10 gallon fish tanks, with an average daily water temperature of 28.5°C, and an average pH of 7.0. The ratio of females to males is maintained at 2 to 1 for optimal embryo production.

The animals are kept in a controlled environmental room, which monitors air temperature and air quality. For mating purposes, daily exposure to light is controlled by an electronic timer that exposes them to a light cycle of 14 hours daylight and 10 hours darkness. Zebrafish embryos are obtained from daily spawnings of the colonies in our facility. To protect the eggs, 13 mm glass marbles are added to the bottoms of the tanks to prevent scavenging of the spawned eggs by adult fish. Eggs are removed from the bottoms of the tanks using a gravity-driven siphon.

Siphoned tank water is discarded and replaced with fresh distilled water supplemented with sea salts (600 mg/L). Once the embryos are collected,

embryos are cultured in embryo rearing medium (ERM) (Westerfield, 1995), until they reach the desired stage. At either the 64-cell blastomere stage or germ-ring stage, embryos are isolated from the general population and grouped according to stage in fresh ERM.

Unfertilized eggs were removed from breeding age adult female zebrafish. Females were anesthetized in tricaine solution for 30 seconds, removed from the solution, their genital pores dried, and the fish laid on their side, gently stroked from front to back along their abdomens, and eggs collected (Westerfield, 1995). Eggs were then water activated in ERM. All embryos were incubated at a constant temperature of 28.5°C.

Treatment of Embryos With Cycloheximide

At the 64-cell and Germ-ring stages, embryos are placed in ERM containing CHX 100μg/ml (Sigma-Aldrich). CHX treated embryos were incubated at 28.5°C up to 8 hrs. Embryos were either immediately processed or groups of 15, 30, or 60 embryos are removed, placed in a 1.5ml Eppendorf® tube, spun down, and snap frozen in crushed dry ice. The snap frozen embryos are held at -85°C until they were needed.

Sequential Image Analysis

Cultured eggs and embryos were viewed with the Olympus SZH10 Research Stereo Microscope and digitally photographed with a NEC NC-15 CCD Color Camera at hourly intervals. All magnifications were 52.5x.

Acridine Orange/ Propidium Iodide Staining

Stock Acridine orange (AO) (Calbiochem) and Propidium iodide (PI) stains were made with ERM to a final working concentration of 100μg/ml. Embryos were collected, washed in ERM, their chorions removed, animal poles isolated, and the animal poles placed into 100 μl ERM with 1 μl of 100 μg/ml acridine orange (AO), and 1 μl of 100 μg/ml propidium iodide (PI), and then incubated at room temperature for 15 min in the dark. Animal poles were washed with ERM, and visualized using a Nikon OPTIPHOT-2 fluorescence microscope with the appropriate filters and photographed with the Pixera 120es Digital Microscope Camera.

Hoechst 33342 Staining

Hoechst 33342 (HOE) (Calbiochem) was prepared in ERM to a final working concentration of 100 μg/ml. Embryos were collected, washed in ERM, and their chorions removed. The animal poles were isolated and placed

into 100 µl ERM with 1 µl of 100 µg/ml Hoechst 33342 (HOE), and then incubated at room temperature for 15 min in the dark. Animal poles were washed with ERM, and visualized using a Nikon OPTIPHOT-2 fluorescence microscope with the appropriate filter and photographed with the Pixera 120es Digital Microscope Camera.

Germ-ring and 64-cell Blastomere Genomic DNA Extraction

The DNA extraction procedure was adapted from an established protocol (Gavrieli *et al.*, 1992). 60 germ-ring stage embryos were placed into a 1.5 ml Eppendorf® tube, spun down briefly and snap frozen. The embryos were allowed to thaw, then 100µl of high EDTA lysis buffer was added, and the sample homogenized. An additional 300µl of lysis buffer was added, re-homogenized, and vortexed. The sample was allowed to sit at room temp to allow for protein solubilization by the detergent. 200µl of phenol:chloroform:isoamyl-alcohol (50:48:2) was added, and the sample vortexed. The sample was then centrifuged for 5 min at 14k rpm at 4°C in a refrigerated microfuge centrifuge. 350µl of upper aqueous phase was collected, and placed into a new Eppendorf® tube. 200µl of choloroform:isoamyl-alcohol (24:1) was added, and the tubes were vortexed. The sample was centrifuged again under the same conditions. 250 µl of the top aqueous phase

was collected and placed into a new Eppendorf®. 100µl of 3M sodium acetate was added, along with 800µl of ethanol, followed by vortexing. The DNA was allowed to precipitate in a -20°C freezer for 30 min. The sample was then centrifuged at 14k rpm for 10 min at 4°C in a refrigerated microfuge centrifuge. The supernatant was decanted, the Eppendorf® turned upside down, and the pellet allowed to dry on a Kimwipe® at RT. Once the pellet was dry 20µl of TE buffer was used to re-suspend the DNA.

DNAzol® Reagent, Genomic DNA Isolation Reagent (Gibco BRL, Grand Island, NY) was used for 64-cell stage blastomeres because of the high content of maternal mRNA found in early embryos. Embryos were rinsed in ERM to remove any debris. Embryos were then placed in a 1.5ml centrifuge tube and spun down. Excess liquid was removed. 300 µl of DNAzol reagent was placed into the 1.5 ml tube, and the sample homogenized. An additional 700 µl of DNAzol® reagent was added into the 1.5 ml tube and the tube was vortexed thoroughly. The sample was left at RT for 5 min, and re-vortexed. The samples were then centrifuged at 14,000 rpm for 20 min at 4°C to pellet the debris. The supernatant was removed and placed into a new 1.5 ml tube. 500 µl of 100 % ethanol was added to the tube, which was vortexed lightly. The sample was left at RT for 5 min. The sample was then centrifuged at 14,000 rpm for 20 min at 4°C to pellet the DNA. The supernatant was

removed and discarded. The DNA pellet was then re-suspended with 10 µl TE buffer. 5 µl loading buffer was added to the sample, which was mixed well before loading onto gels. Samples were run on a 1% agarose gel.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was done on a 1% agarose gel. The gel was prepared by combining 1.0g of agarose (Sigma) with 100ml of 1x TBE, mixing well and boiling in a microwave on high for 4 min. The volume was then returned to 100ml with boiling ddH_20. 10µl of ethidium bromide (10mg/ml) (Sigma) was added, followed by mixing. The solution was then poured into a casting unit and a comb inserted into the unit. The gel was allowed to cool for 30 min before samples were loaded. 1x TBE was used as the running buffer. Ladder style molecular weight markers were used (Sigma), and electrophoresis was done at 120v.

TUNEL Assay

The TUNEL assay was adapted from established protocols (Gavrieli *et al.*, 1992, Yager *et al.*, 1997, Chan *et al.*, 1998). The Fluorescein-FragEL™ DNA FRAGMENTATION DETECTION KIT (Oncogene Research Products, San Diego, CA) was used to perform the TUNEL assay. Embryos were washed in

TBS for 1 min at RT, and any debris removed. Embryos were fixed in 4% formaldehyde overnight at 4°C. They were then twice washed in TBS for 30 min at 4°C. 100 µl Proteinase K Solution was placed into a hybridization chamber well. The embryos were placed into the well and their chorions removed. The embryos were incubated in the Proteinase K Solution for 20 min at RT. Embryos were then twice washed in TBS for 10 min at RT, then incubated in 100 µl 1x TdT Equilibration Buffer for 30 min at RT. The 1X TdT Buffer was then removed, 100 µl TdT Labeling Reaction Mix was added, and the embryos incubated for 90 min at 37°C in the dark. The Reaction Mix was then removed and the embryos immersed in 100 µl TBS for 5 min at RT. As a positive control, embryos were exposed to DNase I (Sigma) before the assay. The enzyme produced an intense TUNEL signal in all nuclei. The DNase I was dissolved in PBS (1µg/ml), and treatment consisted of immersing the sample in DNase I for 10 min at RT. The sample was then rinsed with dH_20. The TUNEL assay has been performed on zebrafish embryos previously, and no mitotic DNA was labeled using this technique (Yager *et al.*, 1997).

Fluorometric Enzyme Assay For Caspase-3 Activity

The assay used is an adaptation from an established protocol (Mirkes *et al.*, 2000). 30 embryos were placed in an Eppendorf® tube and snap frozen in crushed dry ice. 510µl of fluorogenic lysis buffer was added and the sample homogenized. An additional 510µl of buffer was added and the sample re-homogenized. The sample was then vortexed and centrifuged at 14k rpm for 20min in a 4°C refrigerated microfuge centrifuge. 995µl of sample was then removed and placed into a test tube cuvette. 5µl of caspase-3 fluorogenic substrate (5mg/ml) (Bachem) was then added, and the sample was mixed with a Pasteur pipette. Fluorescence readings were taken every 15min, using a 405nm excitation filter and 515nm emission filter. The readings were taken up to 75min and the 60min reading was plotted.

DePsipher Mitochondrial Transmembrane Potential Assay

DePsipher® (Trevigen, Gaithersburg, MD) solutions were prepared as indicated by the manufacturer. Embryos were collected, washed in ERM, and their chorions removed. All animal poles were isolated and placed into 100 µl of DePsipher® Reaction Mixture on a printed diagnostic slide. Animal poles were incubated at 28.5°C for 15 min in the dark. Animal poles were washed with ERM and visualized using a Nikon OPTIPHOT-2 fluorescence

microscope with the appropriate filters and photographed with the Pixera 120es Digital Microscope Camera. Untreated control embryos were processed in the same fashion.

Caspase-3 Live Cell Enzyme Assay

Animal pole cells were collected as above and placed into 100 µl ERM with 5 µl of 5mg/ml Ac-DEVD-AFC, 1 µl of 100 µg/ml propidium iodide, and 1 µl of 100 µg/ml bisbenzimide H 33342, and then incubated at 28.5°C for 60 min in the dark. Animal poles were processed for fluorescence microscopy as above.

Annexin V Staining

Phosphatidylserine localization was assayed with the Vybrant Apoptosis Assay Kit, Alexa Fluor 488 conjugated to annexin V (Molecular Probes, Eugene, OR). Animal poles were collected as above and placed into 100 µl 1X Annexin-Binding Buffer. 1 µl of 100 µg/ml propidium iodide, 1 µl of 100 µg/ml bisbenzimide H 33342, and 5 µl Alexa Fluor 488 annexin V stock were added, and the preparations were incubated at room temperature for 15 min in the dark. They were washed with 1 X Annexin-Binding Buffer and visualized as above.

Western Blot and Electrophoresis

The western blotting and electrophoresis procedure used was adapted from Bollag *et al*., 1996. All western blots were run in the same manner. Kaleidoscope Prestained Standards were used as molecular weight markers (Bio-Rad Laboratories, Hercules, CA). Samples were directly applied to 4-15% BioRad precast Ready gels (BioRad Laboratories), run on a BioRad Mini-PROTEAN II unit at 200v using a BioRad Power Pac 200 power supply (BioRad Laboratories). Immediately after electrophoresis, the gels were overlaid with Immun-Blot PVDF membrane (BioRad Laboratories) which was presoaked in methanol and equilibrated using TBS. The gel and membrane were then covered with two pieces of wet filter paper, placed into the transfer cassette and placed into the BioRad Mini-PROTEAN II blotting unit (BioRad Laboratories). The transfer was run at 100v for 90 min. PVDF membranes were immediately blocked using 3% BSA for 60 min. While the membrane was blocking, the gel was stained with PhastGel Blue R Coomassie stain (Pharmacia Biotech) for 10 min and then destained overnight in destain to visualize transfer efficiency. Following this step, the membrane was washed for 30min in TBS and then the membrane was incubated in Anti-PARP, Bovine, raised in Rabbit (Calbiochem) primary antibody, (15µl in 15ml TBS), overnight at 4^{o}C. The next day the membrane was washed for 30 min with TBS and then placed

in Anti-Rabbit IgG secondary antibody (Sigma-Aldrich) conjugated to horseradish peroxidase (10μl in 15ml TBS), for 2 hrs at 4°C. Following this step, the membrane was washed with TBS for 30 min. The protein was then visualized by reaction with a substrate 3,3" diaminobenzidine tetrahydrochlhoride (DAB) (Sigma-Aldrich). Color development was stopped by rinsing the membrane with ddH_20.

Transmission Electron Microscopy

The protocol for transmission electron microscopy was developed in collaboration with Dr. Louis Trombeta. Embryos were collected, washed in TBS, and fixed with fresh 3% EM grade glutaraldehyde (Sigma-Aldrich) (v/v) for 3 hrs at 4°C. Embryos were then rinsed with TBS, immersed in 1% osmium (Sigma-Aldrich) (v/v) for 1 hr at RT, and rinsed again with TBS. Embryos were then dehydrated in an acetone series (30, 60, 90, and 100%) (v/v) for 10 min at RT. Embryos were then infiltrated in a Araldite/acetone series (1-2, 30 min 1-1, 30 min and 2-1, 60 min), and then infiltrated in pure Araldite for 60 min. Flat embedding molds were filled with fresh Araldite, the embryos placed into the wells, and animal poles positioned toward the cutting point. Sections were cut using a glass knife.

CHAPTER III

Results: Section I

Gross Morphological Effects of Unsuccessful Fertilization on Zebrafish Eggs

To evaluate the gross morphological effects of unsuccessful fertilization in zebrafish eggs, sequential digital recordings were used. In mammals, ova which are not fertilized undergo synchronous degeneration. Based on in vitro studies, researchers have shown that if a postovulatory mouse egg is not fertilized within 24 hrs, it will undergo programmed cell death (Perez *et al.*, 1999). Postmeiotic unfertilized starfish eggs have also been shown to die by apoptosis (Yuce and Sadler, 2001). Thus, it seems that the ovum is programmed to die unless fertilization of the ovum occurs and embryogenesis ensues. Little is known about the mechanism of such degeneration in vertebrates.

In the absence of fertilization, postmeiotic interphase arrest persists for 16-20 hrs, after which starfish eggs synchronously and rapidly die. At the early stages of post-meiotic unfertilized starfish egg death, membrane blebbing, and the beginning of cytoplasmic contraction is observed. During the middle stages of starfish egg death, cytoplasmic darkening, cytoplasmic contraction, and membrane blebbing are more apparent. During the later stages of starfish

egg death, the cytoplasm becomes completely dark, is highly contracted, and the cells begin to fragment (Yuce and Sadler, 2001).

Unfertilized zebrafish eggs die in a synchronous and rapid fashion, displaying many morphological changes. Changes in cytoplasmic clarity were first visible is unfertilized eggs by the 3rd hour post-activation Fig. 1A3. A patch of cytoplasmic darkening was visible just above the yolk sac in the cytoplasm. Other areas of the cytoplasm began to show signs of cytoplasmic darkening by the 5th hour post-activation Fig. 1A5. By the 7th hour post-activation, the entire cytoplasmic compartment was darkened Fig. 1A7. By the 8th hour post-activation all unfertilized eggs had ruptured Fig. 1A8. Unfertilized eggs die at a stage equivalent to 8 hpf embryos in fertilized control embryos, a time when embryos would normally be gastrulating Fig. 1B8. These data suggest that a cell death mechanism of maternal origin is present before fertilization and that this mechanism is activated by the 3rd hour post-activation if the egg is unsuccessfully fertilized.

Figure 1. Sequential digital recording of synchronously dying water activated eggs.

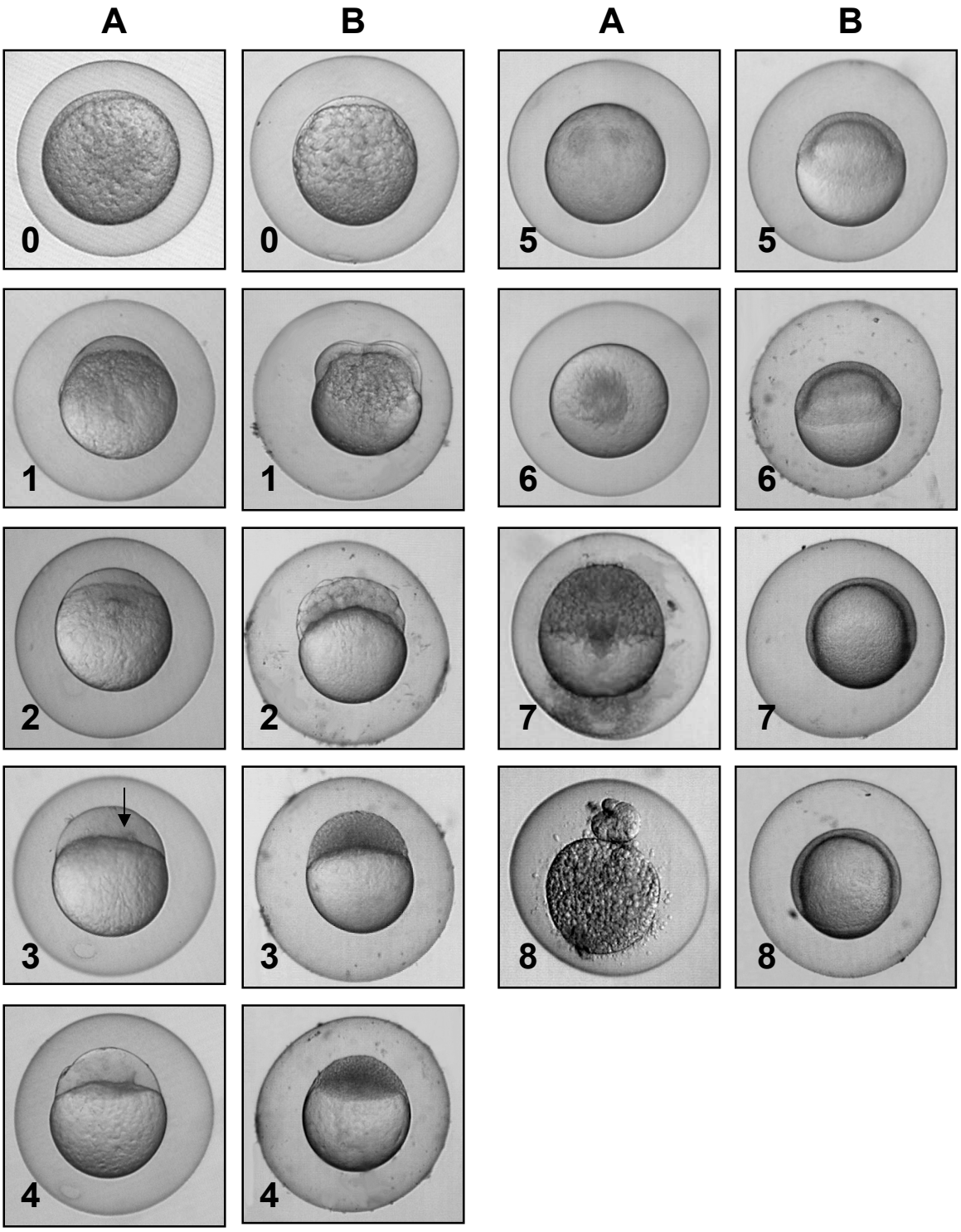


Figure 1. Sequential digital recording of synchronously dying water activated eggs. Zebrafish eggs were isolated from breeding age adult females using a light stroking motion over their abdomens forcing the release of mature eggs. Zebrafish egg death was induced and ensued after 8 hrs by water activation in embryo rearing medium. Cultured eggs were viewed with the Olympus SZH10 Research Stereo Microscope and digitally photographed with a NEC NC-15 CCD Color Camera at hourly intervals. Fertilized control embryos were also digitally photographed. Panels A 0-8 are unfertilized water activated eggs. Panels B 0-8 are fertilized controls. The numbers represent hrs post-fertilization. All magnifications are 52.5x. (A) Panels 0-2, unfertilized egg displaying no signs of cytoplasmic irregularities. Panels 3-5, unfertilized egg begins to show signs of cytoplasmic opacity. Panel 6, unfertilized egg continues to undergo changes in cytoplasmic opacity. Panel 7, unfertilized egg undergoes a change in opacity of entire cytoplasm. Panel 8, unfertilized egg loses cell membrane integrity. (B) Panels 0-8 display normal developmental morphology of fertilized zebrafish eggs.

The Effects of Cycloheximide on 64-cell Blastomere Stage Progression and Survival

To evaluate the effects of cycloheximide in 64-cell blastomeres, blastomere stage progression and survival was analyzed using sequential digital recordings. During the first four rounds of cell division, cell death does not occur in normal mouse blastomeres (Handyside and Hunter, 1986; Weil *et al.*, 1996). Not until the first blastocyst formation does normal cell death occur, predominantly in the inner cell mass, in a variety of species including mouse and human (Handyside and Hunter, 1986; Hardy *et al.*, 1989). Chronic treatment of 4 and 8-cell stage zebrafish blastomeres with aphidicolin and hydroxyurea (inhibitors of DNA synthesis) caused death of the blastomeres when they reach 8-10 hrs of age (Ikegami *et al.*, 1999). If the same treatments were applied to blastomeres between the 16-cell stage and the midblastula transition, most of the blastomeres survive past what would be gastrulation stage in control embryos (Ikegami *et al.*, 1999). Treatment of 2-cell stage *Xenopus* embryos with cycloheximide causes an arrest in cell division within 1 hr of treatment (Hensey and Gautier, 1997). Prolonged exposure of *Xenopus* embryos to cycloheximide at the 2 and 4-cell stage arrested the embryos but did not stimulate an apoptotic response by the embryos until stage 10.5, a time corresponding to the onset of gastrulation (Hensey and Gautier, 1997).

In our hands 64-cell stage zebrafish blastomeres underwent synchronous and rapid death when exposed to cycloheximide. Cycloheximide treated 64-cell stage blastomeres arrest within the 1st hr of exposure Fig. 2A1. By the 2nd hr of treatment, animal pole cells seem to begin to contract, and cell cycle arrest is more obvious when compared to untreated controls Fig. 2A2. After the 3rd hr of treatment shedding of cells from the animal pole has begun to take place Fig. 2A3. By the 4th hr of treatment the entire animal has detached from the vegetal pole, and the animal pole cells continue to lose cell-cell contact Fig. 2A4. The death of the blastomere occurs by the 5th hr with the rupture of many of the animal pole cells Fig. 2A5. 64-cell stage blastomeres die at a stage equivalent to 7 hpf embryos in untreated control embryos, a time when the embryos would normally be gastrulating Fig. 2B7. These data suggest that 64-cell stage embryos are capable of cell death in a rapid fashion when treated with cycloheximide. Sixty-four cell stage embryos do not transcribe new messages. They can only translate proteins from maternal mRNA supplied to them. The fact that 64-cell stage embryos undergo such a rapid cell death suggests that cell death machinery of maternal origin may be present, and that it may be proteolytically activated in the presence of cycloheximide.

Figure 2. Sequential digital recording of synchronously dying cycloheximide treated 64-cell stage blastomeres.

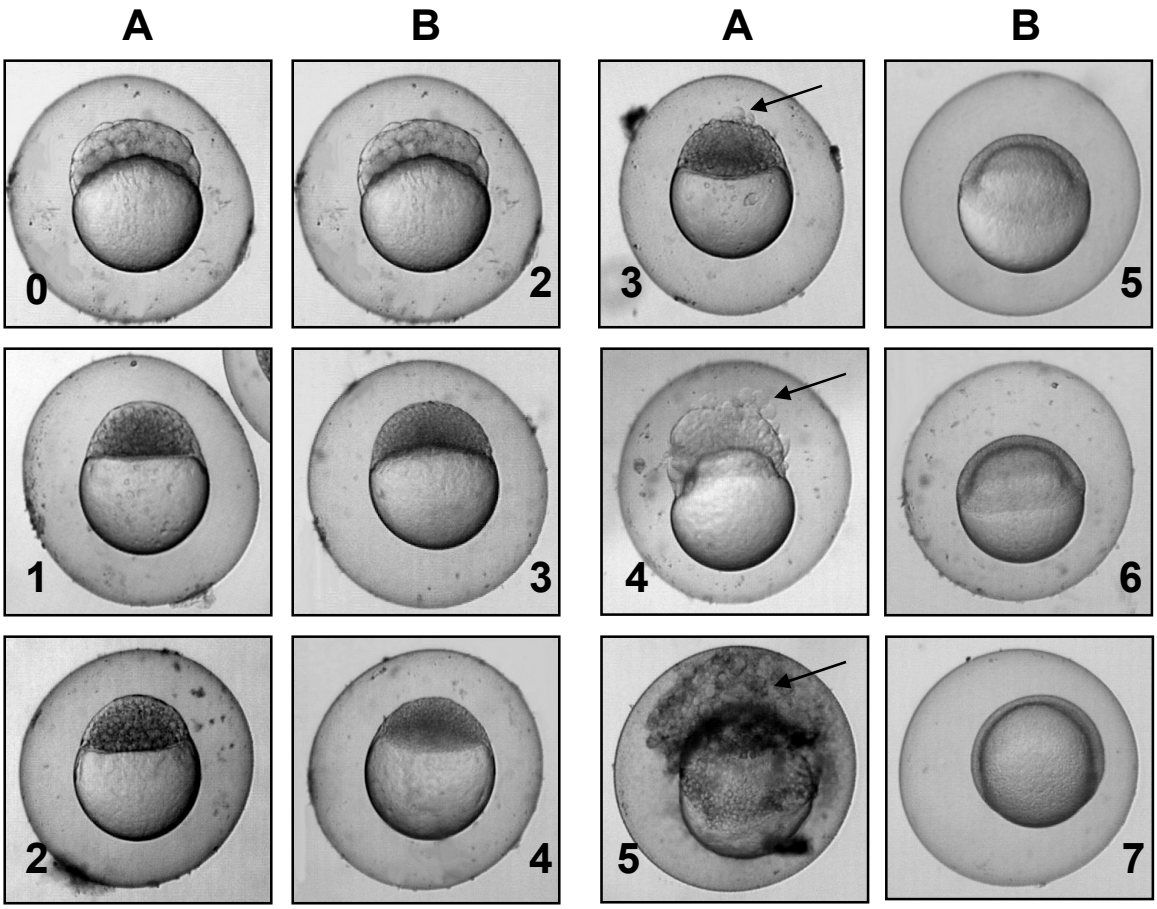


Figure 2. Sequential digital recording of synchronously dying cycloheximide treated 64-cell stage blastomeres. Fertilized zebrafish embryos were obtained from natural spawnings in marbled tanks. Death was induced in 64-cell stage blastomeres by incubation in media with 100 μg/ml of cycloheximide for up to 5 hrs. Cultured blastomeres were viewed with the Olympus SZH10 Research Stereo Microscope and digitally photographed with a NEC NC-15 CCD Color Camera at hourly intervals starting at 2 hpf. Fertilized control embryos were also digitally photographed. Fig. 2 Panels A 0-5 are cycloheximide treated 64-cell blastomeres. Fig. 2 Panels B 2-7 are untreated control embryos, each number representing hpf. All magnifications are 52.5x. (A) Panel 0, 64-cell stage blastomere. Panel 1, the blastomere display signs of cell cycle arrest and stoppage of normal stage progression when compared to the untreated control. Panel 2, blastomere stage progression remains arrested, and the animal pole begins to contract. Panel 3, first signs of cell dissociation and shedding are seen. Panel 4, cell shedding continues with animal pole separating from the vegetal pole. Panel 5, all blastomere cells lyse. (B) Panels 2-7 display normal stage progression of untreated control embryos.

The Effects of Cycloheximide on Germ-ring Embryonic Stage Progression and Survival

To evaluate the effects of cycloheximide on germ-ring stage embryos, embryonic stage progression and survival was analyzed using sequential digital recordings. *Xenopus* embryos treated with cycloheximide prior to the midblastula transition undergo cell cycle arrest and show signs of apoptosis at stage 10.5 whereas embryos treated after the midblastula transition undergo cell cycle arrest, but they do not display signs of apoptosis (Hensey and Gautier, 1997). The first signs of apoptosis in these embryos were evident much later during neurulation at 15.5 hpf, and many of these embryos survive up to 24 hpf, however, their development is abnormal (Hensey and Gautier, 1997).

When zebrafish embryos are treated after the midblastula transition for 1-2 hrs with 20-60 μM camptothecin (a topoisomerase I inhibitor), most nuclei retain a normal appearance, but cell division does suddenly stop (Ikegami *et al.*, 1997a). When treatment with 60 μM camptothecin is prolonged, these embryos remain alive until ~10 hours of age, yet they suddenly die at this time (Ikegami *et al.*, 1999). Treatment of zebrafish embryos with nocodazole (a microtubule destabilizer) after the MBT produces metaphase arrest in the cells of the deep cell layer (Ikegami *et al.*, 1997b). Germ-ring stage embryos undergo synchronous and rapid death when exposed to cycloheximide. The first

obvious effects of cycloheximide treatment in germ-ring stage embryos is a visible decrease in the rate of stage progression in the embryo. Untreated control embryos progress past the shield stage Fig. 3B1 whereas cycloheximide treated embryos progress to about the shield stage Fig. 3A1. By the 2nd hr of treatment there is an obvious arrest in stage progression; changes in cell adhesion and changes in embryonic morphology are seen, and the germ-ring margin begins to disappear Fig. 3A2. Embryos at the 3rd hr display extensive embryo movement in the animal pole direction, possibly due to embryo contraction and loss of cell adhesion to the vegetal pole Fig. 3A3. By the 4th hr the animal pole begins to detach from the vegetal pole, a sign of extensive loss in cell-cell attachment Fig. 3A4. During the 5th hr post-treatment, the animal pole cells begin to display extensive loss of cell-cell contact Fig. 3A5. By the 6th hr post-treatment, the cytoplasm of most of the animal pole cells has opacified Fig. 3A6. Death of the embryo is obvious by the 7th hr as most animal pole cells have ruptured Fig. 3A7. These data suggest that cycloheximide treated germ-ring stage embryos contain cell death machinery that responds to treatment with the protein synthesis inhibitor in a rapid fashion. The origin of this machinery is presumptively zygotic as maternally inherited proteins and mRNA no longer influence the embryo.

Figure 3. Sequential digital recording of synchronously dying cycloheximide treated germ-ring stage embryos.

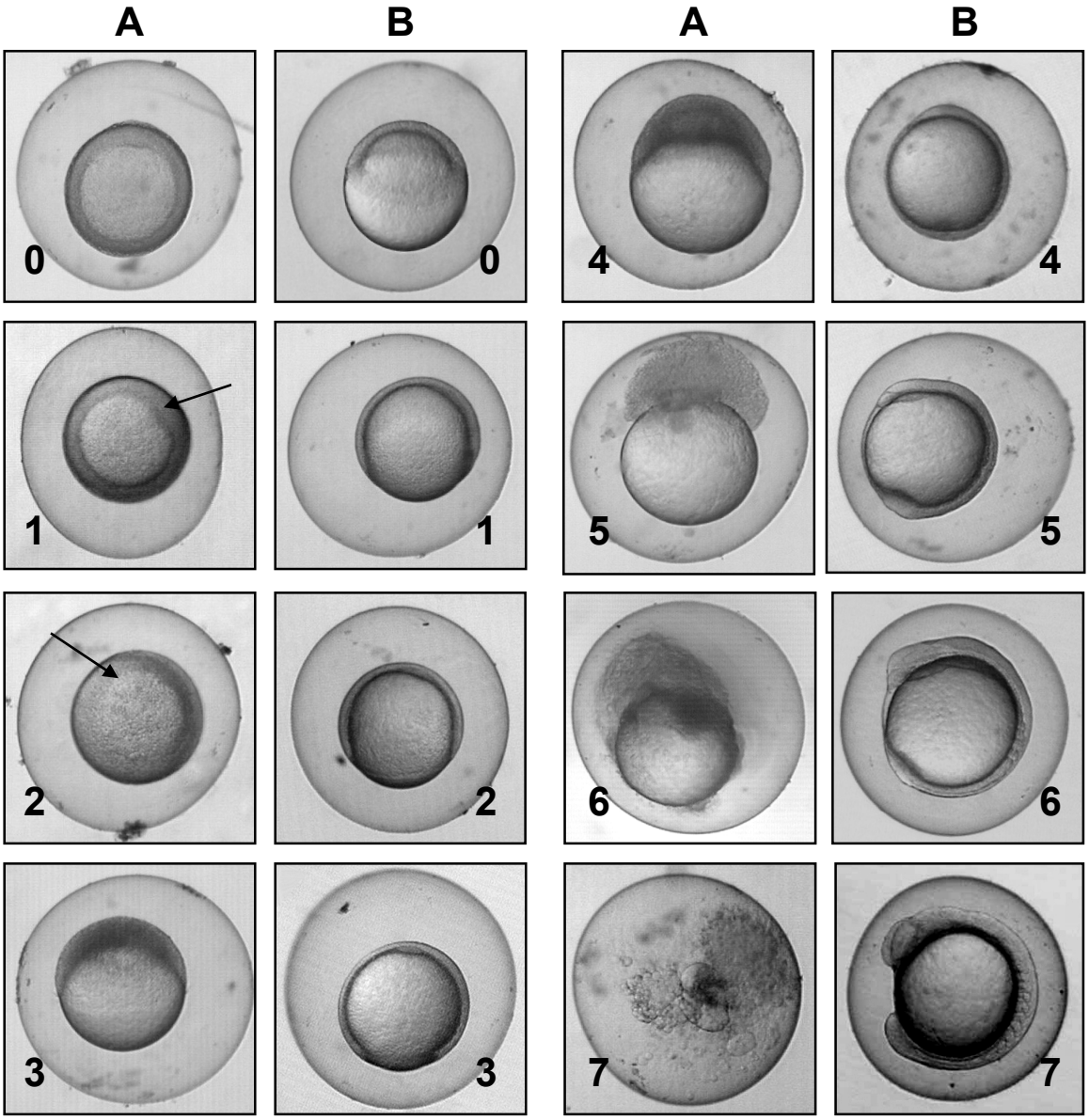


Figure 3. Sequential digital recording of synchronously dying cycloheximide treated germ-ring stage embryos. Fertilized zebrafish embryos were obtained from natural spawnings in marbled tanks. Death was induced in germ-ring stage embryos by incubation in media with 100 μg/ml of cycloheximide for up to 7 hrs. Cultured embryos were viewed with the Olympus SZH10 Research Stereo Microscope and digitally photographed with a NEC NC-15 CCD Color Camera at hourly intervals starting at $5^{2/3}$ hpf. Control embryos were also digitally photographed. All magnifications are 52.5x. Panels A 0-7 are cycloheximide treated germ-ring stage embryos, with each number representing the hours of treatment. Panels B 0-7 are untreated control embryos representing normal development. (A) Panel 0, germ-ring stage embryo at 0 hr treatment. Panel 1, germ-ring stage embryo shows signs of retardation in stage progression at the shield stage, arrow points to embryonic shield. Panel 2, changes in cell adhesion and embryonic morphology are seen, the germ-ring margin begins to disappear, arrow points to area where the sharp margin should be. Panel 3, extensive movement in the animal pole direction by the embryo is seen after 3 hrs treatment, possibly due to embryo contraction. Panel 4, the animal pole of the embryo begins to detach from the vegetal pole after 4 hrs treatment. Panel 5, the animal pole begins displaying extensive cell-cell contact loss. Panel 6, the cytoplasm of most of the cells has darkened in

the animal pole. Panel 7, most if not all of the cells in the animal pole have lysed. (B) Panels 0-7 display normal stage progression of untreated control embryos.

The Effects of Cycloheximide Treatment on Zebrafish Early Embryo Survival Rates, and the Effects of Unsuccessful Fertilization on Zebrafish Egg Survival Rates

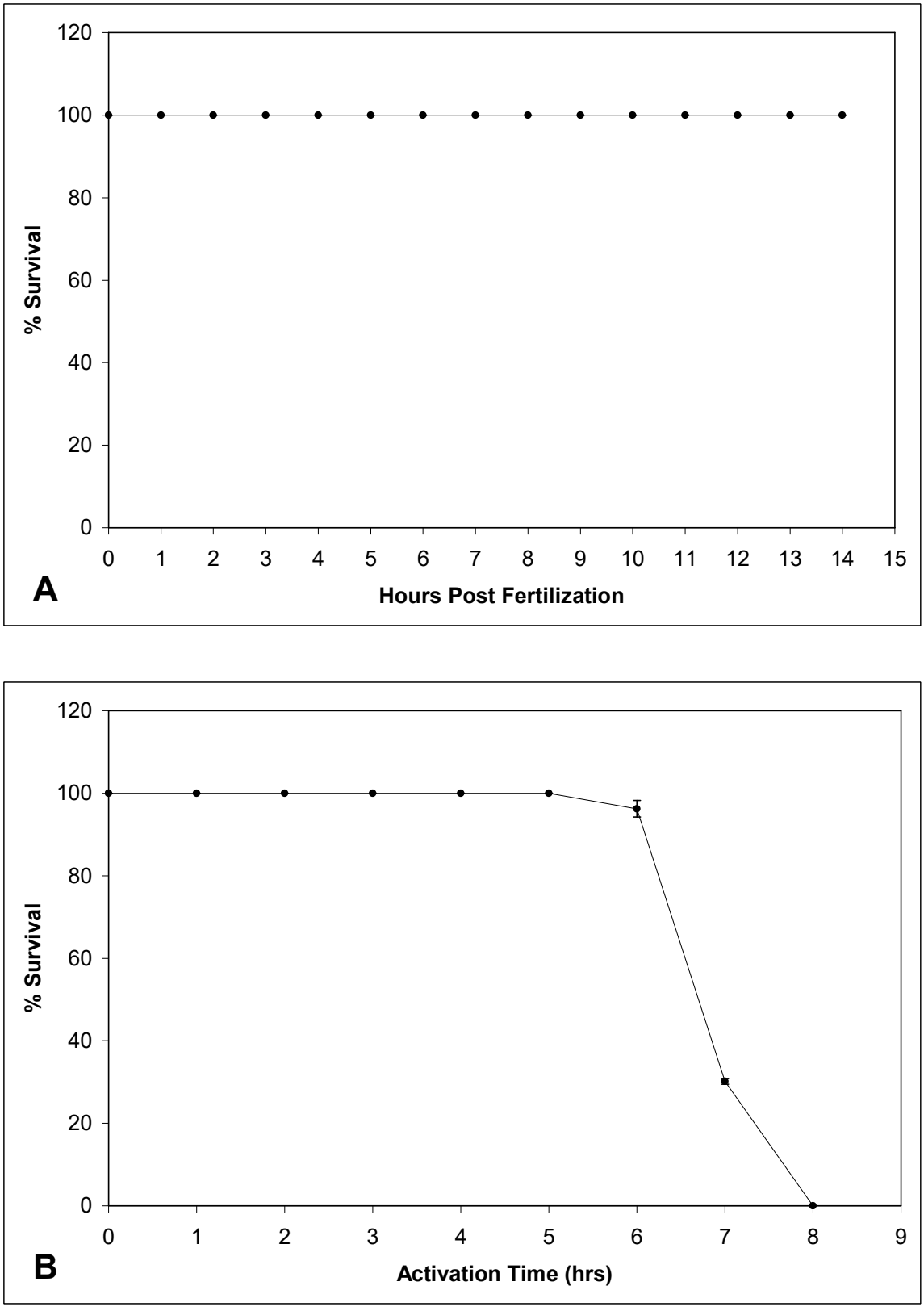
To examine the effects of cycloheximide treatment on zebrafish early embryo survival rates, populations of embryos at the 64-cell blastomere and germ-ring stages were incubated in 100 μg/ml cycloheximide (Sigma-Aldrich, Saint Louis, MO) for up to 8 hrs. To examine unsuccessful fertilization on zebrafish egg survival rates, populations of zebrafish eggs were isolated from breeding age adult zebrafish females, and water activated in fresh embryo rearing medium. Percent survival refers to the % of surviving embryos in a population. 64-cell blastomere and germ-ring stage embryos displaying less than 25% visibly ruptured cells were scored as alive. Unfertilized eggs displaying an intact cell membrane were scored as alive.

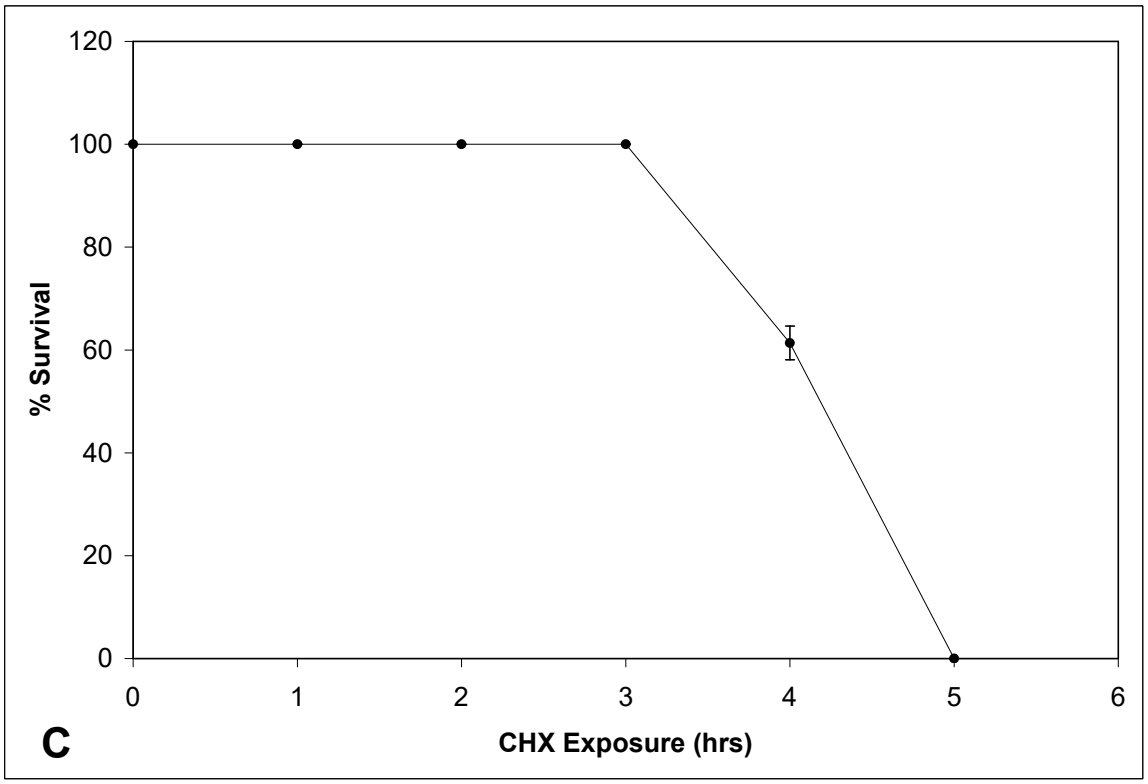
At the cellular level, cycloheximide blocks the translation of messenger RNA on cytosolic ribosomes, but it does not inhibit organelle protein synthesis (Obrig *et al.*, 1971). Treatment of 2-cell stage *Xenopus* embryos with cycloheximide (a protein synthesis inhibitor) causes an arrest in cell division within 1 hr of treatment, due to the inhibition of cyclin synthesis (Hensey and Gautier, 1997). When cycloheximide was applied continuously at concentrations that block 97% of protein synthesis, *Xenopus* embryos arrested

cell division but did not die until a time when they would normally be gastrulating (Hensey and Gautier, 1997).

These data suggest that populations of cycloheximide treated embryos and unfertilized eggs die in a highly reproducible fashion. Chronic exposure to cycloheximide causes death of all 64-cell stage blastomeres and all germ-ring stage zebrafish embryos. Untreated control embryos develop normally and most if not all survive to reach segmentation Fig. 4A. Unfertilized zebrafish eggs die in a synchronous fashion, with a few deaths occurring 6 hours post-activation, and the rest of the deaths occurring in the 7th and 8th hrs Fig. 4B. Cycloheximide treated 64-cell stage blastomeres die in a synchronous fashion, with 40% of the embryos dying in the 4th hr and the remainder by the 5th hr Fig. 4C. Cycloheximide treated germ-ring stage embryos die in reproducible fashion, with 10% of the embryos dying in the 5th hr, another 20% dying in the 6th hr, and the rest dying in the 7th and 8th hr. Whether the embryos and eggs die through apoptosis or necrosis can not be determined by this method. All samples studied appear to have a mechanistic mode of death with similar morphologies seen in deaths induced by other agents in zebrafish and *Xenopus* embryos.

Figure 4. Percent survival curve for zebrafish eggs and early embryos.




120
100
80
60
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% Survival
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CHX Exposure (hrs)
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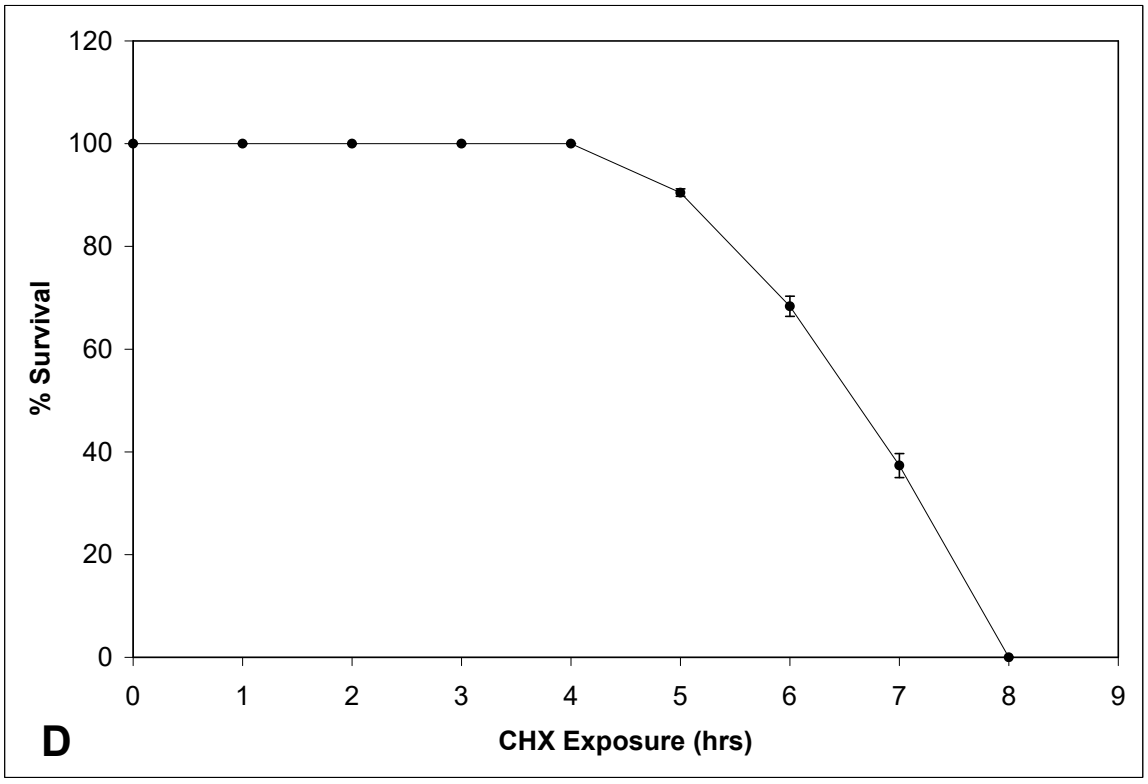
120
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% Survival
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CHX Exposure (hrs)
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Figure 4. Percent survival curve for zebrafish eggs and early embryos. Large populations of fertilized zebrafish embryos were obtained from natural spawnings in marbled tanks. Zebrafish eggs were isolated from breeding age adult females using a light stroking motion over their abdomens forcing the release of unlaid eggs. Cell death was induced as previously described. Cultured eggs and embryos were checked at hourly intervals and the number of surviving embryos recorded. Dead eggs and embryos were removed and discarded. (A) Untreated control embryos collected at the 2-cell stage generally had a 100% survival rate when allowed to develop to 14 hpf. (B) Unfertilized zebrafish eggs began to die at about the 6th hr of culture but a few survived up to the 8th hr after water activation, a time corresponding to midgastrulation in successfully fertilized control embryos. (C) Treatment of 64-cell stage blastomeres with cycloheximide caused embryonic death at about the 4th hr of culture, equivalent to the 6th hpf in untreated controls. By the 5th hr of treatment all embryos were judged to be dead, a time corresponding to midgastrulation in untreated control embryos. (D) Treatment of germ-ring stage embryos with cycloheximide caused embryonic death beginning the 5th hr of culture, equivalent to 11th hpf in untreated controls. By the 8th hr of treatment all embryos were judged to be dead, a time corresponding to the early segmentation period in untreated control embryos.

Summary of Section I

Water activated unfertilized zebrafish eggs die within 8 hrs. The cause of the death is unknown, but populations of activated eggs die in a synchronous fashion. All of the dying eggs displayed signs of death seen in other animals, including the opacification of the cytoplasm. The death of the water-activated unfertilized eggs in our experimental system is similar to that mentioned by Kane and Kimmel, 1993, though they did not elaborate or develop this aspect of their study.

Treatment of 64-cell stage blastomeres and germ-ring stage embryos with cycloheximide caused the rapid death of both types of embryos within 8 hrs. Whether both embryos died via the same pathways was not evident. Both embryonic deaths share some similar themes, cell cycle arrest, shedding of animal pole cells, and separation of the animal pole from the vegetal pole. These deaths also shared similarities with published observations of zebrafish cleavage stage embryos and gastrulating embryos treated with other cell death inducers.

It appears that induction of cell death in zebrafish early embryos gives rise to morphologies seen in many other animal model systems. Whether the gross ultrastructural morphologies seen here are caused by the same factors seen in other model systems is unclear.

Results: Section II

Viability of Cycloheximide Treated 64-cell Blastomeres and Germ-ring Stage Embryos

To analyze the viability of cycloheximide treated 64-cell blastomeres and germ-ring stage embryos the acridine orange/propidium iodide Live/Dead assay was used. One of the features distinguishing dead from live cells is the loss of transport function and often even the loss of the structural integrity of the plasma membrane. Acridine orange (AO) exhibits metachromatic fluorescence that is sensitive to DNA conformation, making it useful for detecting apoptotic cells (Darzynkiewicz, 1994). AO is a cell-permeant nucleic acid intercalating dye that emits green fluorescence when bound to dsDNA and red fluorescence when bound to ssDNA or RNA.

Because the intact membrane of live cells excludes charged dyes such as propidium iodide (PI), short incubation with this dye results in selective labeling of dead cells, while live cells show minimal uptake (Darzynkiewicz *et al*., 1992). The PI exclusion analysis is a very common cell viability assay. Viewed by fluorescence microscopy, live cells stain lightly with AO and exclude PI. Apoptotic cells stain heavily with AO due to the increased binding of AO to fragmented DNA. Both late apoptotic cells and necrotic cells allow PI to pass through the cell membrane staining the DNA red, indicating a loss of plasma membrane integrity.

Both 64-cell and germ-ring stage embryos maintain cell membrane integrity until sudden cell lysis . Untreated control 64-cell stage blastomeres stain lightly with AO and PI, indicative of normal chromatin structure and plasma membrane integrity Fig. 5A and B. Cycloheximide treated blastomeres exhibited similar AO and PI staining after 4 hrs treatment Fig. 5C and D. 64-cell blastomeres are able to maintain plasma membrane integrity up to an hour before the animal pole cells rupture.

Germ-ring stage untreated control embryos maintain plasma membrane integrity as displayed by the light AO and PI staining Fig. 5E and F. Cycloheximide treated germ-ring stage embryos exhibited intense AO staining Fig. 5G, yet they displayed light PI staining Fig. 5H. The fact that germ-ring stage embryos treated with cycloheximide stain intensely with AO indicates that the cells are apoptotic. One of the features of apoptosis is maintenance of plasma membrane integrity until late in the process. These data indicate that plasma membrane integrity is maintained in both types of treated embryos until they rupture suddenly. This sudden rupturing could be due to the fact that the embryos are in fresh water, and any change in osmotic balance can cause sudden rupturing of plasma membranes.

Figure 5. Determination of embryonic viability by acridine orange/propidium iodide, Live/Dead assay in cycloheximide treated 64-cell blastomeres and germ-ring stage embryos.

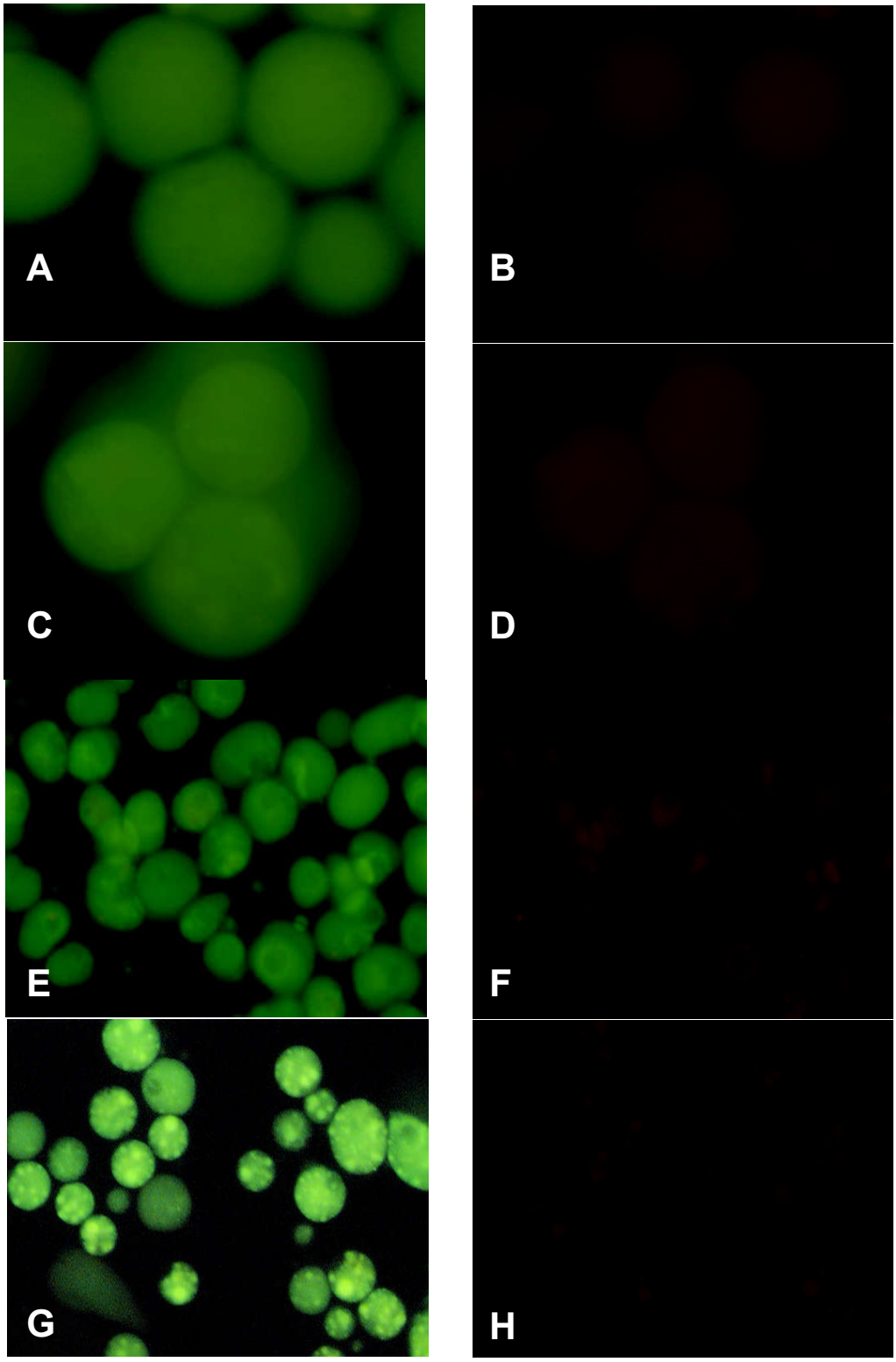


Figure 5. Determination of embryonic viability by acridine orange/propidium iodide, Live/Dead assay in cycloheximide treated 64-cell blastomeres and germ-ring stage embryos. Cell death was induced in zebrafish 64-cell blastomeres and germ-ring stage embryos by incubation with 100 μg/ml of cycloheximide for 4 and 7 hrs. Embryos were processed as in chapter II part D. Animal poles were washed with ERM and visualized using a Nikon OPTIPHOT-2 fluorescence microscope with the appropriate filters and photographed with the Pixera 120es Digital Microscope Camera. Untreated control embryos were processed in the same fashion. A-D are 64-cell blastomeres, E-H are germ-ring stage embryos. A, C, E, and G are stained with AO; B, D, F, and H are stained with PI. A, B and E, F are untreated controls; C, D and G, H are cycloheximide treated. All magnifications are 200x. (A) Untreated control 64-cell blastomeres display light staining with AO. (B) Untreated control 64-cell blastomeres exclude PI. (C) Cycloheximide treated 64-cell blastomeres display light staining with AO. (D) Cycloheximide treated 64-cell blastomeres exclude PI. (E) Untreated control germ-ring stage embryos display light staining with AO. (F) Untreated control germ-ring stage embryos exclude PI. (G) Cycloheximide treated germ-ring stage embryos display extensive staining of chromatin aggregates with AO. (H) Cycloheximide treated germ-ring stage embryos exclude PI.

Nuclear and Chromatin Morphology of Cycloheximide Treated 64-cell Blastomeres and Germ-ring Stage Embryos

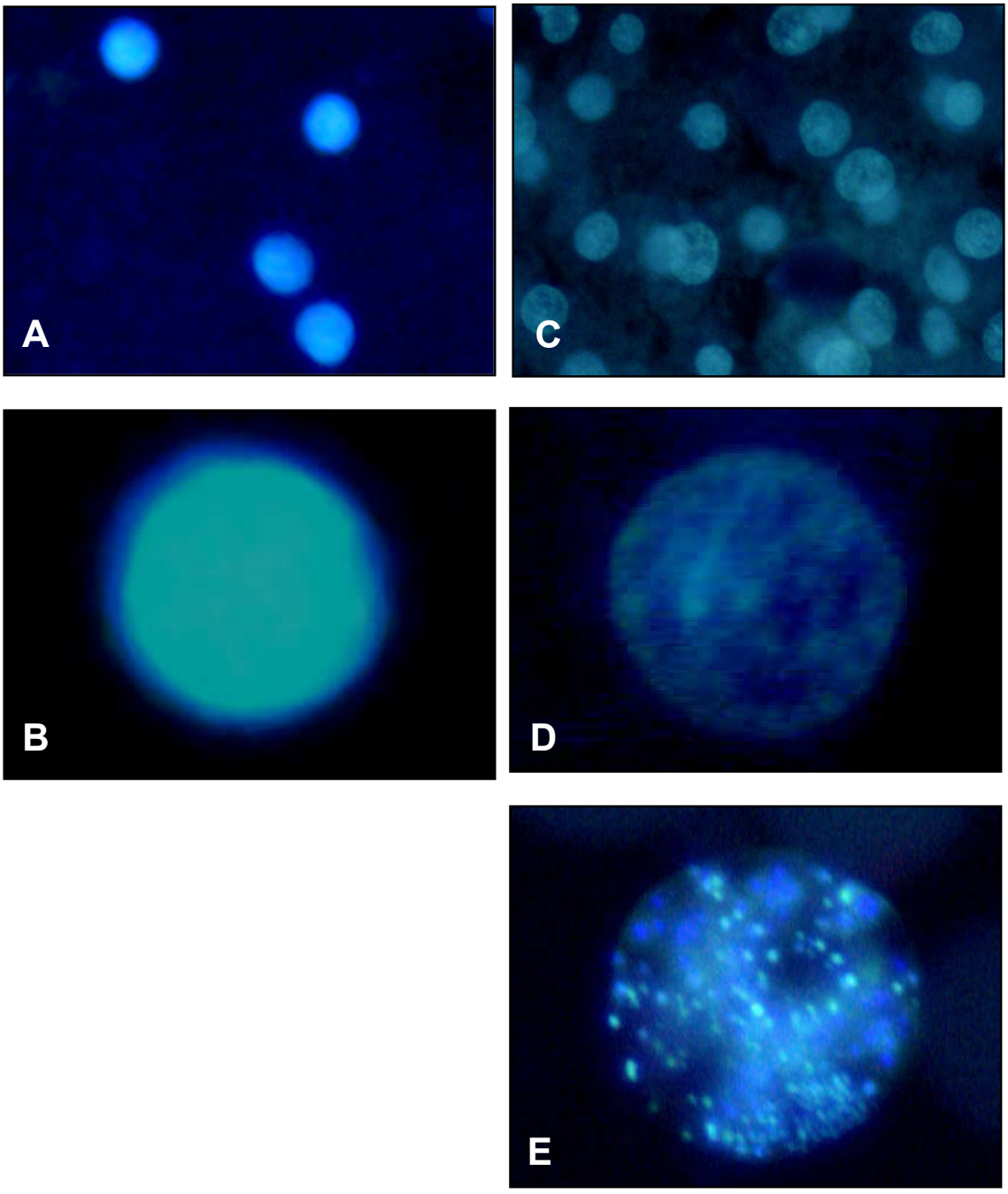
We used vital staining with the DNA binding dye Hoechst 33342 to analyze the nuclear and chromatin morphology of cycloheximide treated 64-cell blastomeres and germ-ring stage embryos. The Hoechst family of dyes consist of benzimidazole derivatives that emit blue fluorescence at 460 nm when excited by UV light at 346 nm. Hoechst 33342 is a cell-permeable adenine-thymidine specific fluorescent stain, but it does not intercalate. During apoptosis, nuclear chromatin fragments into aggregates that stain brightly with fluorescent DNA dyes. Condensed nuclear chromatin gathers at the nuclear membrane and fragmentation of the nucleus then begins with the formation of micronuclei.

Apoptotic and necrotic nuclei display different morphologies when viewed using electron microscopy. During necrotic cell death, mild clumping of nuclear chromatin can occur (Trump *et al.*, 1981). The clumping of nuclear chromatin evident in the early stages of injury becomes more marked with the onset of cytoplasmic degeneration during necrosis. One of the main differences between apoptotic and necrotic chromatin clumping is that necrotic chromatin clumps do not significantly redistribute. In addition, the aggregates that form during necrosis differ in that they do not bud to form discrete, membrane bound fragments.

Cycloheximide-induced 64-cell blastomeres lack characteristic apoptotic chromatin clumping and nuclear changes, yet germ-ring stage embryos display characteristic chromatin clumping and morphological nuclear changes when treated with cycloheximide. 64-cell stage blastomeres exposed to cycloheximide do not display characteristic chromatin clumping seen during apoptosis Fig. 6C and D. The nuclei seen in the dying blastomeres are reminiscent of ghost nuclei which are found predominantly in necrotic cells. A few cells with apoptosis-like chromatin clumps were identified in dying blastomeres Fig. 6E. The reason for this morphology is unknown given the predominantly necrotic looking nuclei in the blastomere.

Cycloheximide treated germ-ring stage embryos display characteristic apoptotic nuclei and chromatin Fig. 6I, J, and K. Convolution of the nuclear membrane and the formation of chromatin clumps confirms that germ-ring stage embryos contain the machinery to execute apoptosis. These data indicate that germ-ring stage embryos display classic apoptotic nuclear changes when exposed to cycloheximide, but rather that 64-cell blastomeres rarely produce apoptotic looking nuclei, instead they mainly produce necrotic looking nuclei. These data are consistent with the idea that there may be maternal inhibition of cell death in blastomere stage embryos but not in gastrulating embryos. In addition, it is known that inhibition of apoptosis via inhibition of caspase activation leads to a necrotic death *in vivo* (Chautan *et al.*, 1999).

Figure 6. Morphological analysis of nuclear fragmentation and chromatin condensation with Hoechst 33342 in cycloheximide treated 64-cell blastomeres and germ-ring stage embryos.



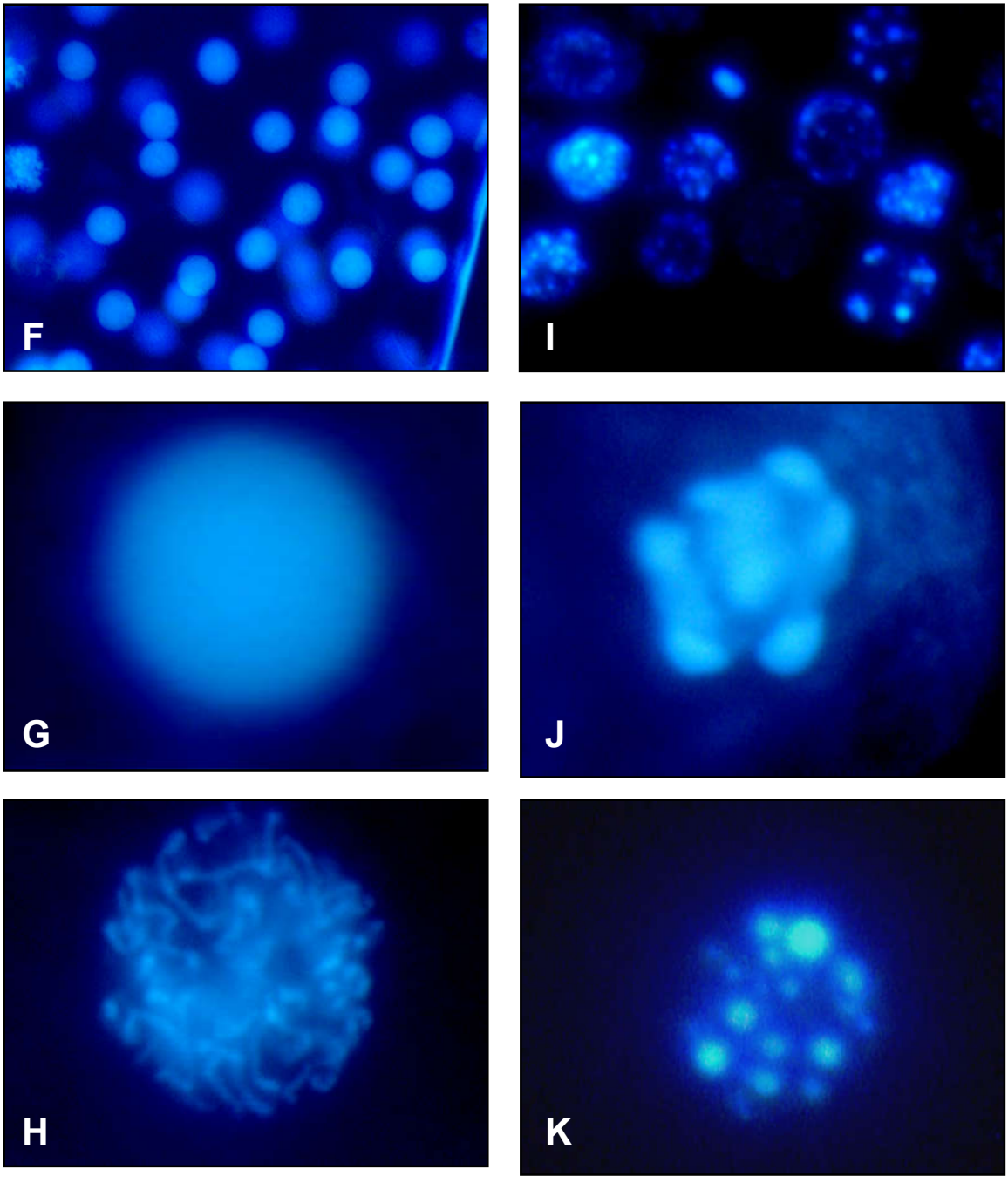

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Figure 6. Morphological analysis of nuclear fragmentation and chromatin condensation with Hoechst 33342 in cycloheximide treated 64-cell blastomeres and germ-ring stage embryos. Apoptosis was induced in zebrafish 64-cell blastomeres and germ-ring stage embryos by incubation with 100 μg/ml of cycloheximide for 4 hrs. Embryos were collected, washed in ERM, their chorions removed, animal poles isolated, and placed into 100 μl ERM with 1 μl of 100 μg/ml Hoechst 33342 (HOE), and then incubated at room temperature for 15 min in the dark. Animal poles were washed with ERM, and they were visualized using a Nikon OPTIPHOT-2 fluorescence microscope with the appropriate filter and photographed with the Pixera 120es Digital Microscope Camera. Untreated control embryos were processed in the same fashion. A-E are 64-cell blastomeres, F-K are germ-ring stage embryos. (A) Untreated control blastomere displaying normal HOE staining, 200x (B) High magnification view of an untreated control blastomere, 1000x (C) Cycloheximide treated 64-cell blastomere displays weak HOE staining, indicating a necrosis-like denaturation of DNA, 200x (D) High magnification view of 64-cell blastomere with ghost-like nuclei after cycloheximide treatment, 1000x (E) Isolated cells with condensed chromatin can be found in 64-cell blastomeres treated with cycloheximide, 400x (F) Untreated control germ-ring stage embryos displaying normal HOE staining, 200x (G) High magnification

image of untreated control germ-ring embryo, 1000x (H) High magnification image of a mitotic cell displaying chromosome staining with HOE, 1000x (I) Cycloheximide treated germ-ring stage embryos display a chromatin clumping morphology characteristic of apoptosis and micronuclei formation, 200x (J) High magnification image of an apoptotic germ-ring stage nucleus displaying extensive chromatin clumping and the generation of nuclear membrane convolutions, 1000x (K) Image of a germ-ring stage cell that has many micronulei in its cytoplasm, a late event in apoptosis, 400x

The Extent of DNA Fragmentation Generated During the Death of Cycloheximide Treated Embryos

The primary objective of this experimenter was to correlate the nuclear morphology seen in cycloheximide treated animal pole cells with the occurrence of DNA fragmentation. To analyze the extent of DNA fragmentation generated during the death of cycloheximide treated embryos, the terminal deoxynucleotidyl transferase (TdT)-mediated dNTP-fluorescein nick end labeling (TUNEL) assay was performed (Gavrieli *et al.*, 1992). During apoptosis, the nucleus undergoes extensive DNA fragmentation. This fragmentation is the result of single and double strand breaks generated by an endonuclease known as Caspase Activated DNase (CAD) (Enari *et al.*, 1998). Apoptotic endonucleases not only affect cellular DNA by producing the classical DNA ladder, but also generate free 3'-OH groups at the ends of these fragments (Gavrieli *et al.*, 1992).

The TUNEL assay is specific in that only the cells that are apoptotic are labeled and stained. TdT is used to incorporate fluorescein conjugated deoxynucleotides at the sites of DNA breaks. The incorporation of the first nucleotide then allows the extension of a chain of fluorescein conjugated nucleotides amplifying the signal generated by the labeling. The TUNEL assay allows apoptotic cells to be identified before morphological changes can be seen in nuclear chromatin.

Germ-ring stage embryos display extensive TUNEL staining, yet 64-cell stage blastomeres lack TUNEL staining following cycloheximide treatment. Cycloheximide treatment of germ-ring stage embryos stimulates the generation of extensive strand breaks and fragmentation in the genomic DNA of the embryos Fig. 7A. Untreated control germ-ring stage embryos display no TUNEL staining Fig. 7B. Cycloheximide treated 64-cell stage blastomeres display no TUNEL staining Fig. 7C, revealing the lack of significant genomic DNA fragmentation. Untreated control blastomeres display no TUNEL staining, revealing healthy genomic DNA Fig. 7D. These data indicate that germ-ring stage embryos have and can use the machinery required for extensive DNA fragmentation, yet 64-cell blastomeres do not display this capability.

Figure 7. Fragment end labeling of nuclear DNA fragmentation in cycloheximide treated embryos.

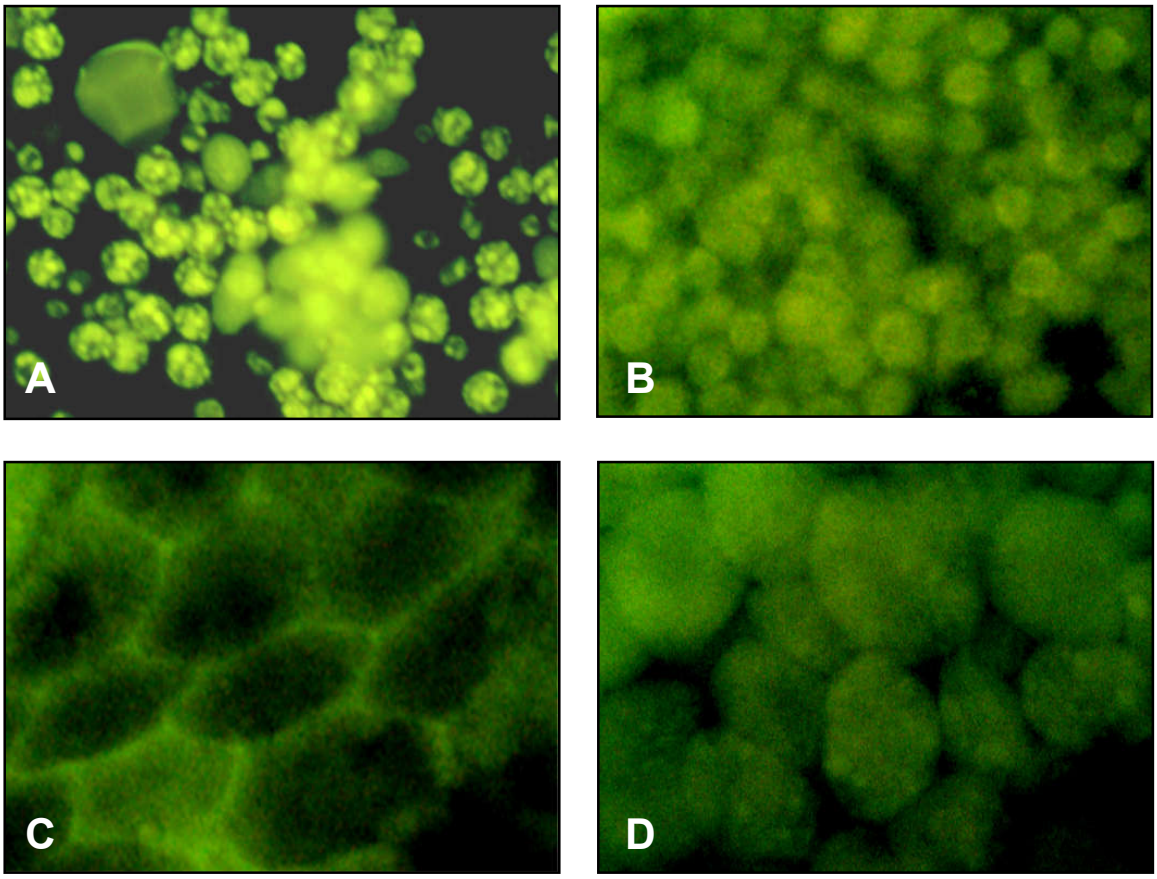


Figure 7. Fragment end labeling of nuclear DNA fragmentation in cycloheximide treated embryos. Apoptosis was induced in zebrafish 64-cell and germ-ring stage embryos by incubation with 100 μg/ml of cycloheximide for 4 hrs. The Fluorescein-FragEL™ DNA FRAGMENTATION DETECTION KIT was used to perform the TUNEL assay (Oncogene Research Products, San Diego, CA). The embryos were processed as directed in the materials and methods. All magnifications are 200x. A and B are germ-ring stage embryos; C and D are 64-cell stage embryos. A and C are cycloheximide treated; B and D are untreated controls. (A) Extensive TUNEL staining is seen in germ-ring stage embryos treated with cycloheximide, revealing extensive DNA strand break formation. Chromatin condensation can also be seen in visibly apoptotic animal pole cells. (B) Untreated control germ-ring stage embryos lack any visible staining above expected background levels. (C) 64-cell stage embryos treated with cycloheximide do not undergo DNA strand break formation before death. (D) Untreated control 64-cell stage embryos lack any visible staining above expected background levels.

The Extent of Internucleosomal DNA Fragmentation in the Death of Cycloheximide Treated 64-cell Blastomeres and Germ-ring Stage Embryos

The most striking biochemical event in apoptosis is the cleavage of genomic DNA between nucleosomes that initially produces 50- to 200-kb segments and fragments in multiples of approximately 185 bp (Wyllie, 1980; Oberhammer *et al*., 1993). This phenomenon is most commonly analyzed by agarose gel electrophoresis, which measures DNA fragmentation in nuclear extracts showing the typical DNA ladder pattern (Compton and Cidlowski, 1986). DNA fragmentation is observed in various tissues and cells during apoptosis. Internucleosomal DNA fragmentation occurs extensively in vivo, including during the formation of interdigital tissues of the embryonic chick leg bud (Garcia-Martinez *et al*., 1993). To analyze the extent of internucleosomal DNA fragmentation in the death of cycloheximide treated 64-cell blastomeres and germ-ring stage embryos, agarose gel electrophoresis of genomic DNA was performed.

DNA fragmentation factor-45 (DFF45)/inhibitor of caspase-activated DNase (ICAD) exists as a complex with a 40-kDa endonuclease termed DFF40/caspase activated DNase (CAD) that promotes apoptotic DNA fragmentation (Enari *et al.,* 1998). Caspase-3 initiates apoptotic DNA

fragmentation by proteolytically cleaving DFF45/ ICAD, which allows the release of active DFF40/CAD (Enari *et al.*, 1998).

Germ-ring stage embryos display extensive DNA laddering when treated with cycloheximide, but DNA laddering is absent in treated 64-cell blastomeres. Cycloheximide treatment of germ-ring stage embryos produces extensive laddering by the 2nd hr of treatment, and continues to produce smaller oligonucleosomal fragments up to the 8th hr Fig. 8A. 64-cell stage blastomeres do not display any laddering associated with apoptosis, evident by the lack of DNA in the molecular weight region where internucleosomal DNA fragmentation is characteristically displayed Fig. 8B. When compared to germ-ring stage embryos exposed to cycloheximide for the same amount of time, extensive internucleosomal DNA laddering can be seen in the 1400 to 500 bp regions Fig. 8C whereas this laddering is not seen in blastomeres treated for the same amount of time with cycloheximide.

These data suggest that 64-cell stage embryos are not capable of executing internucleosomal DNA fragmentation when exposed to cycloheximide, perhaps due to an inhibition of fragmentation or the lack of functioning machinery. This is not the case for germ-ring stage embryos, which produce extensive internucleosomal DNA fragmentation in response to cycloheximide. The gastrulating embryos contain the functional machinery for internucleosomal DNA fragmentation.

Figure 8. Agarose gel electrophoresis analysis of internucleosomal DNA fragmentation in cycloheximide treated embryos.

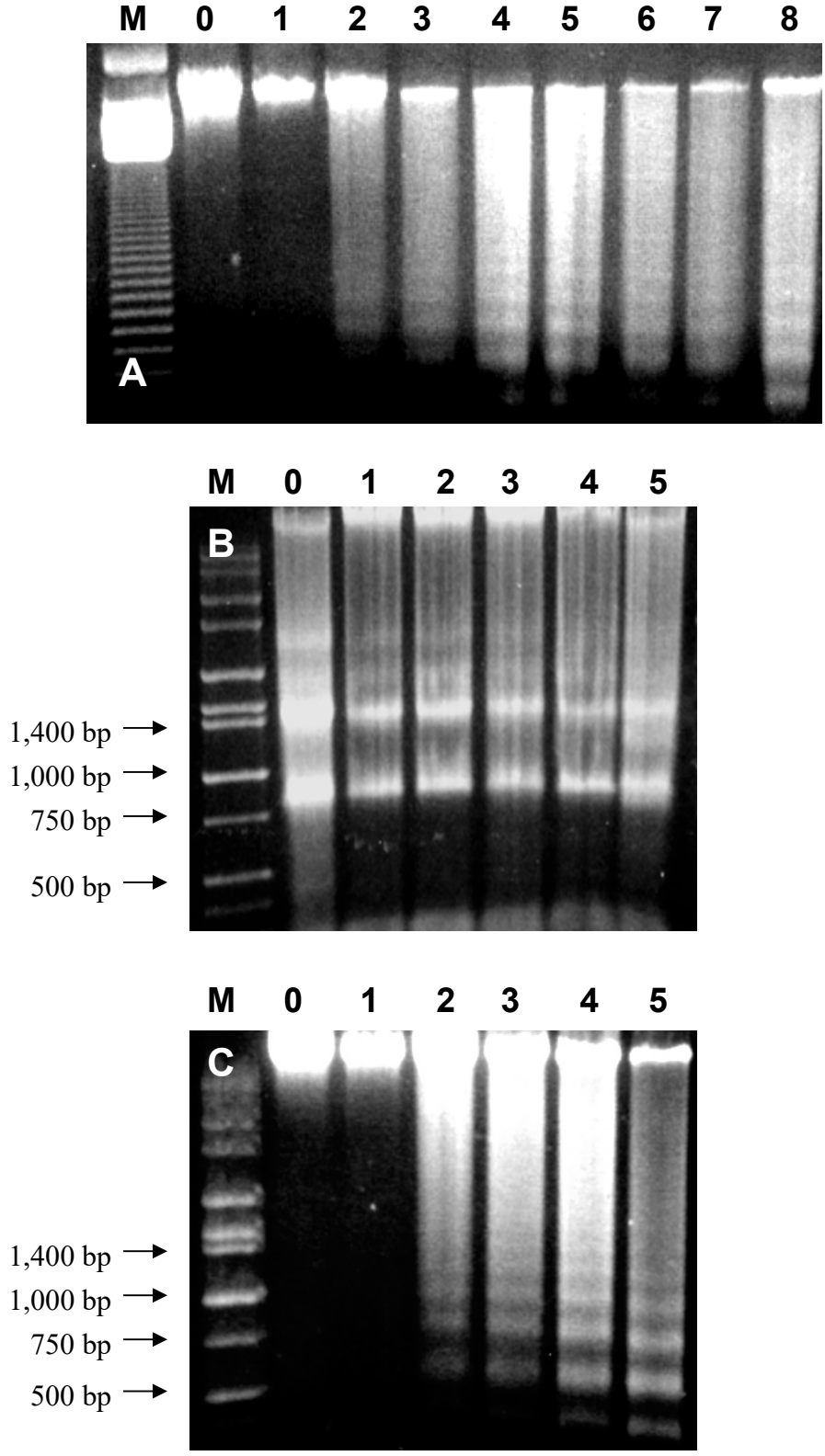


Figure 8. Agarose gel electrophoresis analysis of internucleosomal DNA fragmentation in cycloheximide treated embryos. Cell death was induced in 64-cell blastomeres and germ-ring stage embryos by incubation with 100 μg/ml of cycloheximide for up to 8 hrs. Sets of 120, 64-cell blastomeres and sets of 60, germ-ring stage embryos were collected hourly, washed in PBS, placed into 1.5ml Eppendorf® tubes, spun down at 5,000 rpm, and snap frozen in crushed dry ice. Genomic DNA was extracted from 64-cell stage blastomeres using the DNAzol® Reagent, Genomic DNA Isolation Reagent (Gibco BRL, Grand Island, NY) protocol, and for germ-ring stage embryos phenol-chloroform extraction of genomic DNA was performed. DNAzol® was used for 64-cell stage embryos because of the excessive amount of maternal mRNA found in phenol-chloroform extracted genomic DNA. For experimental procedures see materials and methods. (A) Germ-ring stage embryos exposed to cycloheximide display extensive internucleosomal DNA fragmentation. (B) 64-cell stage blastomeres exposed to cycloheximide do not display characteristic internucleosomal DNA fragmentation ladder. (C) Second agarose gel demonstrating ladder fragment sizes in germ-ring stage embryos, bands which are not present in 64-cell stage blastomeres exposed to cycloheximide.

The Activation of the Proenzyme Form of Caspase-3

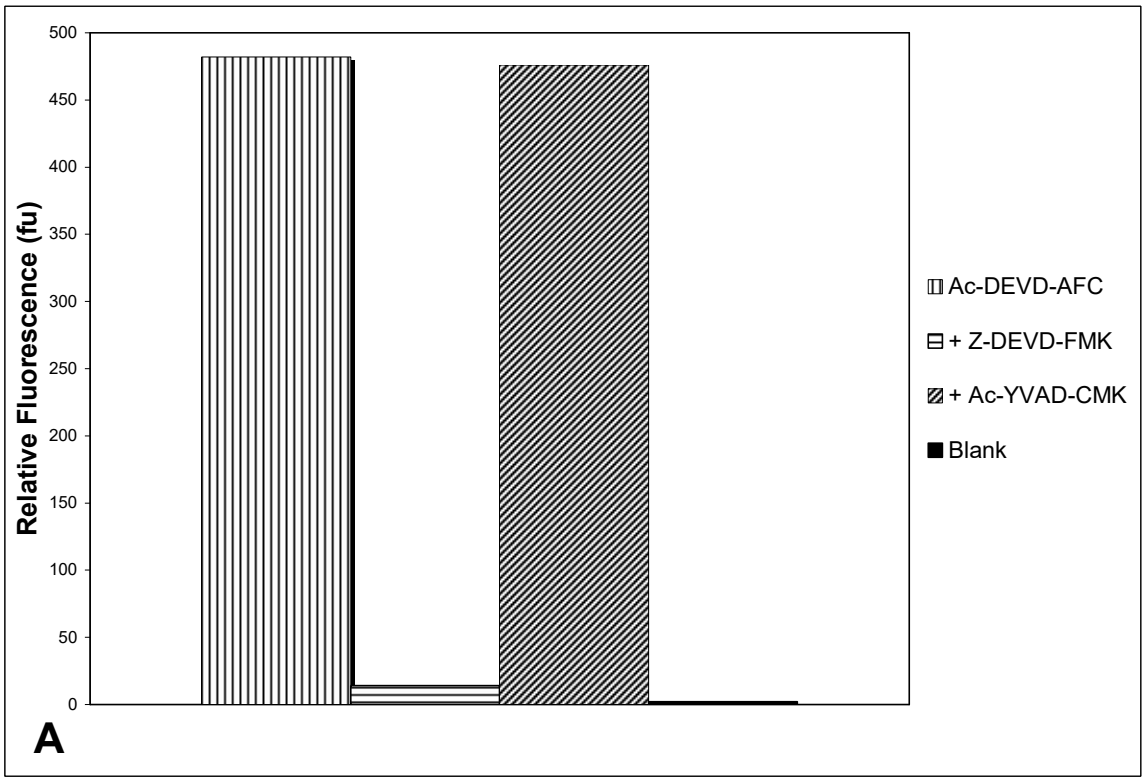
To study the activation of the proenzyme form of caspase-3, a fluorogenic enzyme assay with cytosolic extracts was used to analyze caspase-3 activation. The activation of cysteine proteases from the caspase family is a crucial initial component of the apoptosis machinery in cell death (Yuan *et al.*, 1993). Inhibition of caspase activity in B lymphocytes has been shown to induce a switch in cell death mode from apoptosis to necrosis (Lemaire *et al.*, 1998). Caspases are not the only form of enzyme involved in apoptosis, as many nucleases and protein kinases also participate in the demise of the cell. Yet the active form of one or more caspases is required for the characteristic morphology associated with apoptosis. The regulation of caspase activity occurs predominantly at the level of proenzymes processing and maturation. Dormant caspase proenzymes are converted to catalytically competent heterodimeric proteases by cleavage at Asp-x bonds.

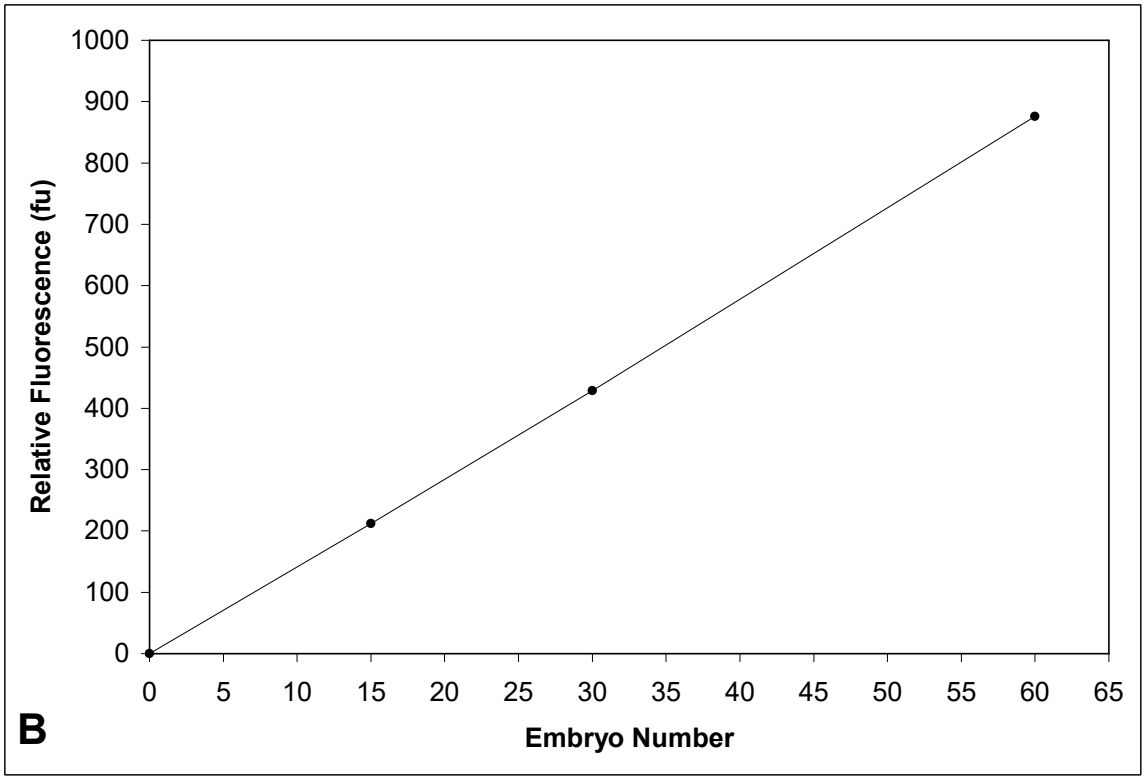
The events that occur as a result of caspase-3 activation are as follows: a halt in cell cycle progression; the disabling of homeostatic and repair mechanisms; the initiation of detachment of the cell from its surrounding tissue structures, disassembly of structural components; and translocation of cell surface markers for engulfment by other cells such as macrophages.

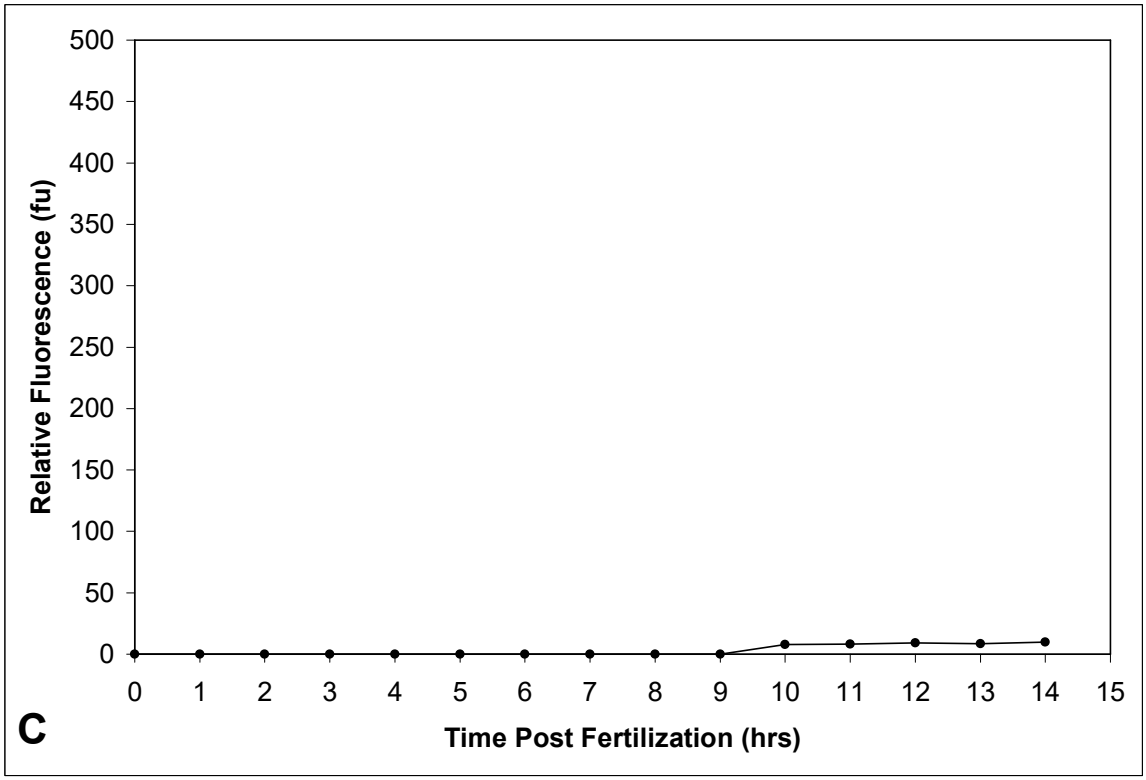
The caspase-3 protease is activated during the deaths of all embryos regardless of the final form of death. Water activated unfertilized zebrafish

eggs successfully activate caspase-3 by the 5th hr of culture Fig. 9D, and the activity climbs sharply by the 7th hr. Cycloheximide treated 64-cell stage blastomeres also successfully activate caspase-3 by the 3rd hr of incubation Fig. 9E, increasing its activity two fold by the 5th hr. Cycloheximide treated germ-ring stage embryos rapidly activate caspase-3 by the 2nd hr of incubation Fig. 9F, increasing in activity ten-fold by the 7th hr of incubation. The activation of caspase-3 is an irreversible event, which could eventually be the mode of egg, 64-cell blastomere, and germ-ring embryonic death, given the proper amount of time to complete its effector role.

Figure 9. Measurement of caspase-3 activity in dying eggs, 64-cell stage blastomeres, and germ-ring stage embryos.



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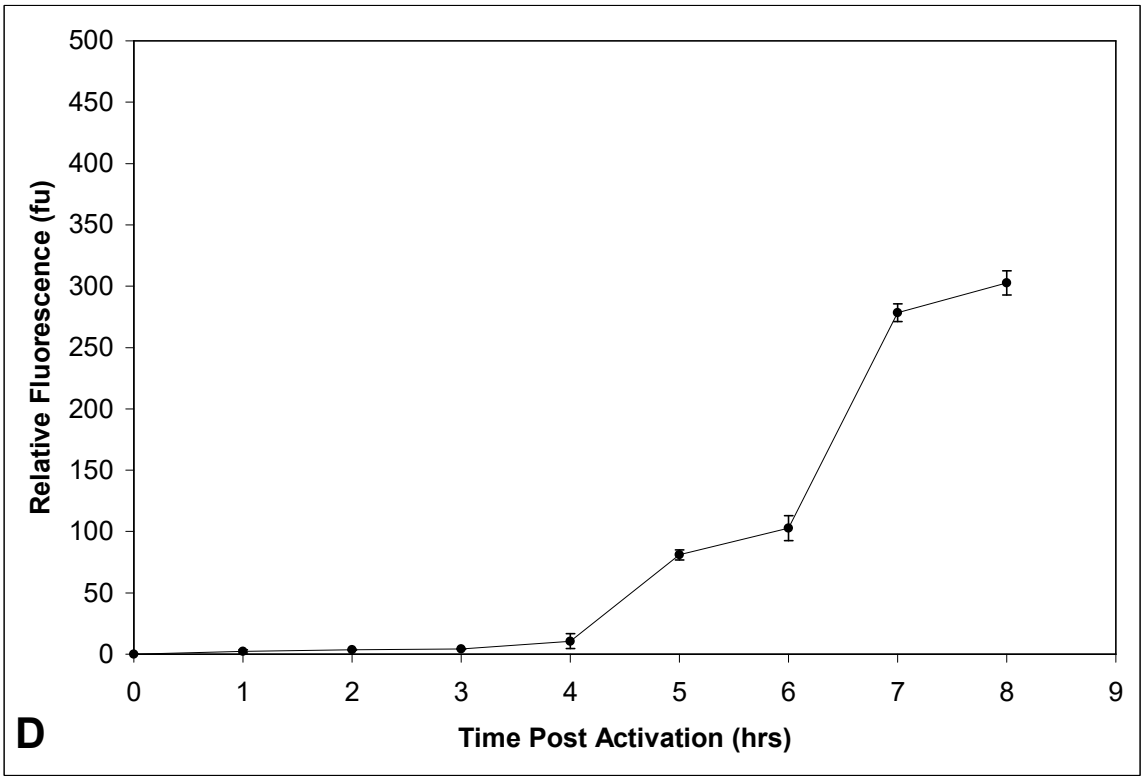
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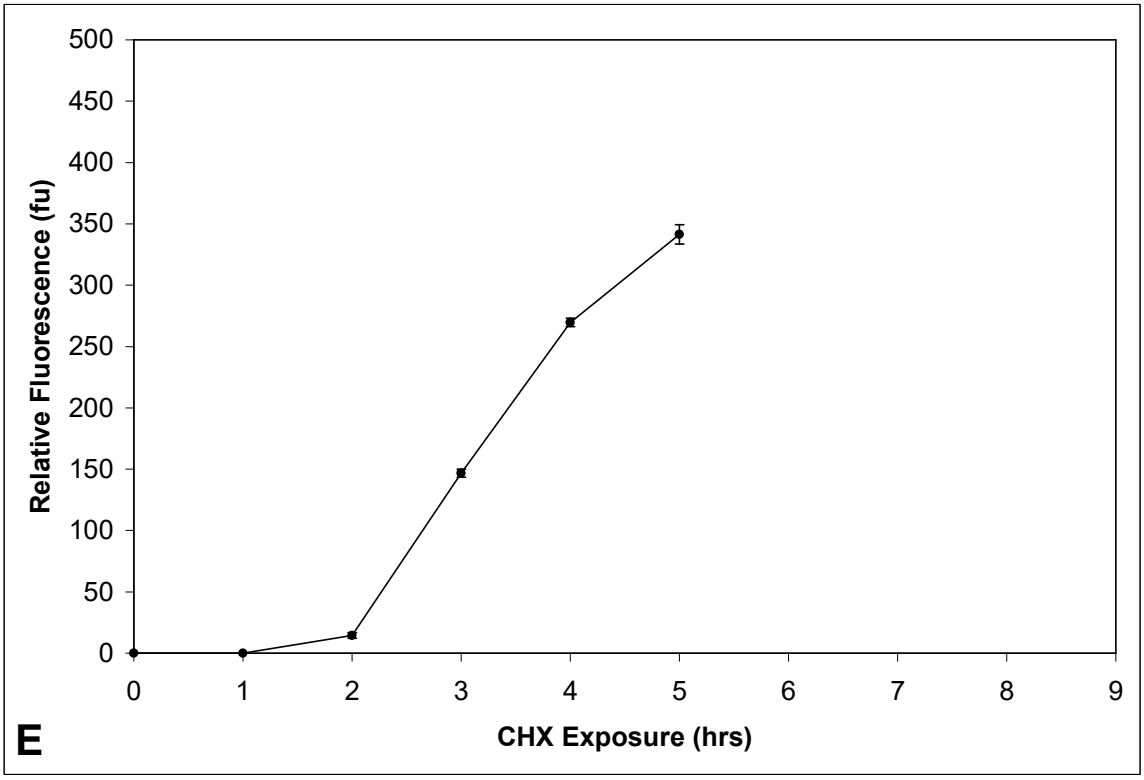
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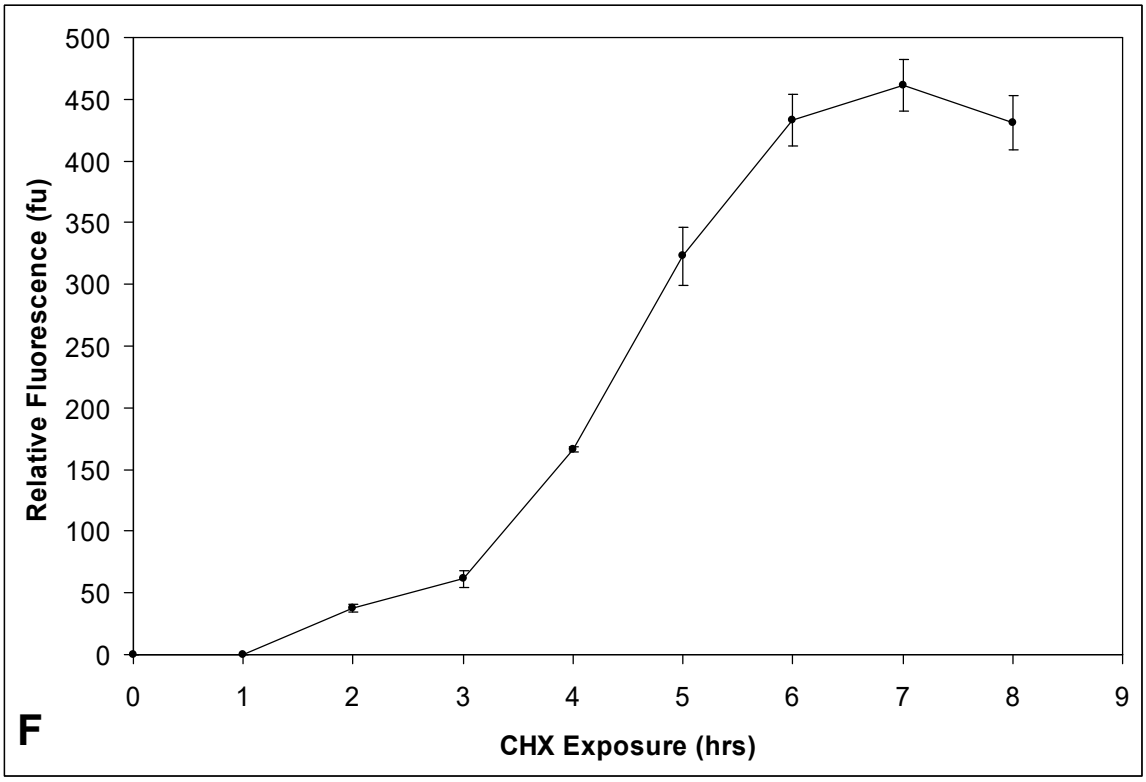
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CHX Exposure (hrs)
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Figure 9. Measurement of caspase-3 activity in dying eggs, 64-cell stage blastomeres, and germ-ring stage embryos. Pools of 30 fertilized zebrafish embryos were obtained from natural spawnings in marbled tanks. The last sample collection was done at a time where all eggs, blastomeres, and embryos were visibly dead in a population. (A) The specificity of the enzyme assay is demonstrated when an inhibitor of caspase-3, Z-DEVD-FMK, diminishes the relative activity of the substrate Ac-DEVD-AFC, and the use of a inhibitor of caspase-1, Ac-YVAD-CMK, does not diminish this activity. (B) The linearity of the enzyme assay in our system demonstrates that doubling the embryo number consistently produces a doubling in relative fluorescence. (C) Untreated control embryos display little to no caspase-3 activity until the embryos reach the end of gastrulation/beginning of segmentation. (D) Water activated zebrafish eggs activate caspase-3 after 4 hrs of culture in ERM, eventually stimulating massive activation. (E) 64-cell stage blastomeres activate caspase-3 after 2 hrs of cycloheximide treatment, rapidly stimulating massive activation. (F) Germ-ring stage embryos activate caspase-3 after 3 hrs of cycloheximide treatment, gradually stimulating massive activation.

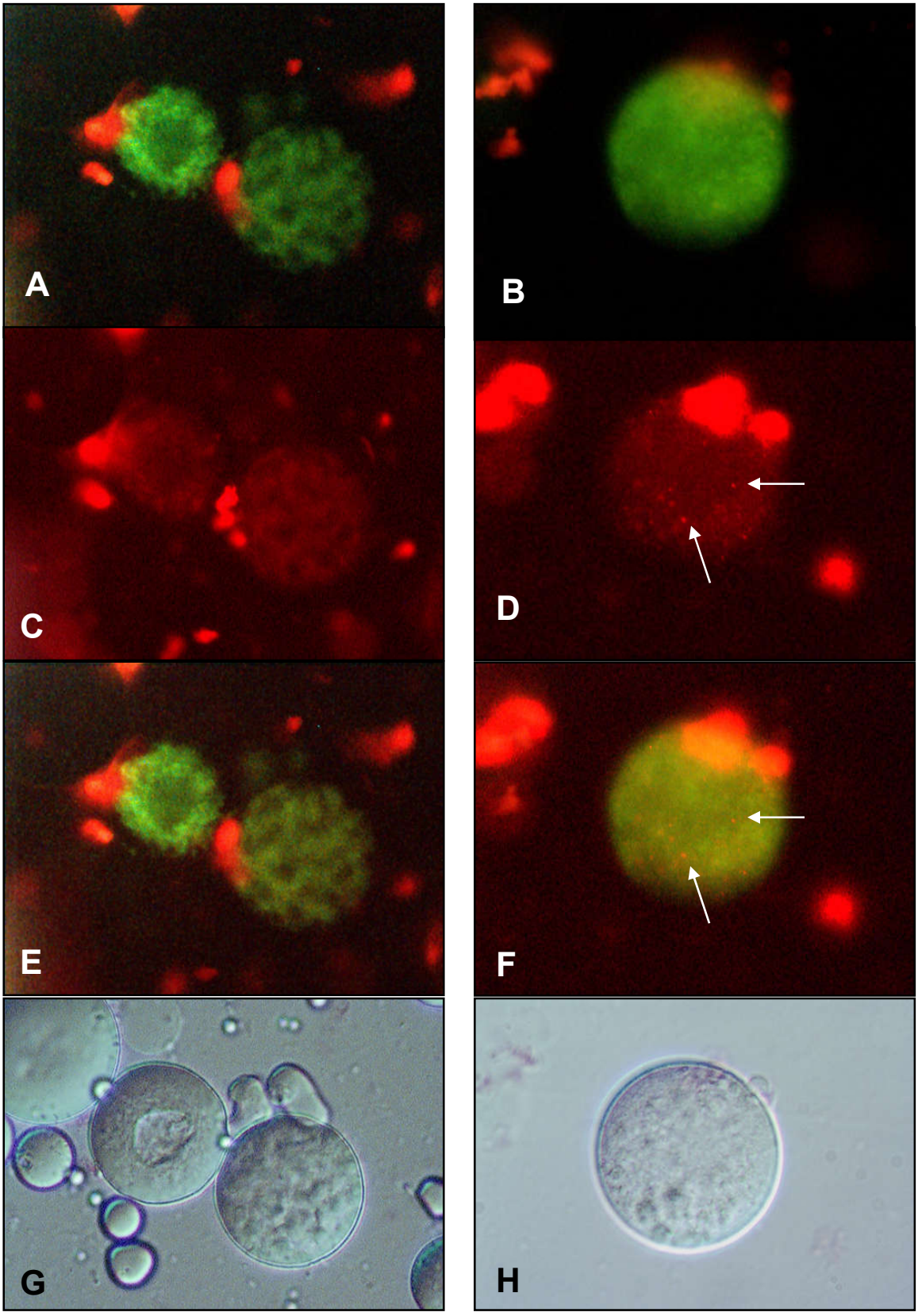
Mitochondrial Transmembrane Potential in Cycloheximide Treated 64-cell Stage Blastomeres and Germ-ring Stage Embryos

Mitochondrial control of apoptosis has been studied extensively in recent years. Energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane. This accumulation of energy in healthy cells creates a mitochondrial transmembrane potential, called $\Delta\psi_m$, that enables the cell to drive the synthesis of ATP. Disruption of $\Delta\psi_m$ has been shown to be one of the first intracellular changes following the onset of apoptosis. This drop in $\Delta\psi_m$ is responsible for a defect of maturation of mitochondrial proteins synthesized in the cytoplasm (Mignotte *et al.*, 1990), cessation of mitochondrial translation, and an uncoupling of the oxidative phosphorylations (Vayssiere *et al.*, 1994).

To analyze the mitochondrial transmembrane potential in cycloheximide treated 64-cell stage blastomeres and germ-ring stage embryos, the DePsipher® assay was used. The DePsipher® assay consists of a lipophilic cation that can be used as a mitochondrial activity marker. This lipophilic cation aggregates upon membrane polarization forming an orange-red fluorescent compound. If the potential is disturbed, the dye cannot access the transmembrane space and remains or reverts to its green monomeric form.

64-cell stages blastomeres lose their mitochondrial $\Delta\psi_m$, yet germ-ring stage embryos retain their mitochondrial $\Delta\psi_m$ after cycloheximide treatment. 64-cell stage blastomeres exposed to cycloheximide display a collapse in mitochondrial $\Delta\psi_m$ by the 4th hour of treatment Fig. 10C, in comparison to untreated control blastomeres, which display significant numbers of mitochondria with intact $\Delta\psi_m$ Fig. 10D. Germ-ring stage embryos exposed to cycloheximide display intact mitochondrial $\Delta\psi_m$ by the 4th hr of treatment Fig. 10K, as they do in untreated control embryos Fig. 10L. These data reveal differences in energy production, as revealed by $\Delta\psi_m$ in 64-cell blastomeres and germ-ring stage embryos which may affect the mode of cell death.

Figure 10. Analysis of mitochondrial transmembrane potential in cycloheximide treated 64-cell stage blastomeres and germ-ring stage embryos.



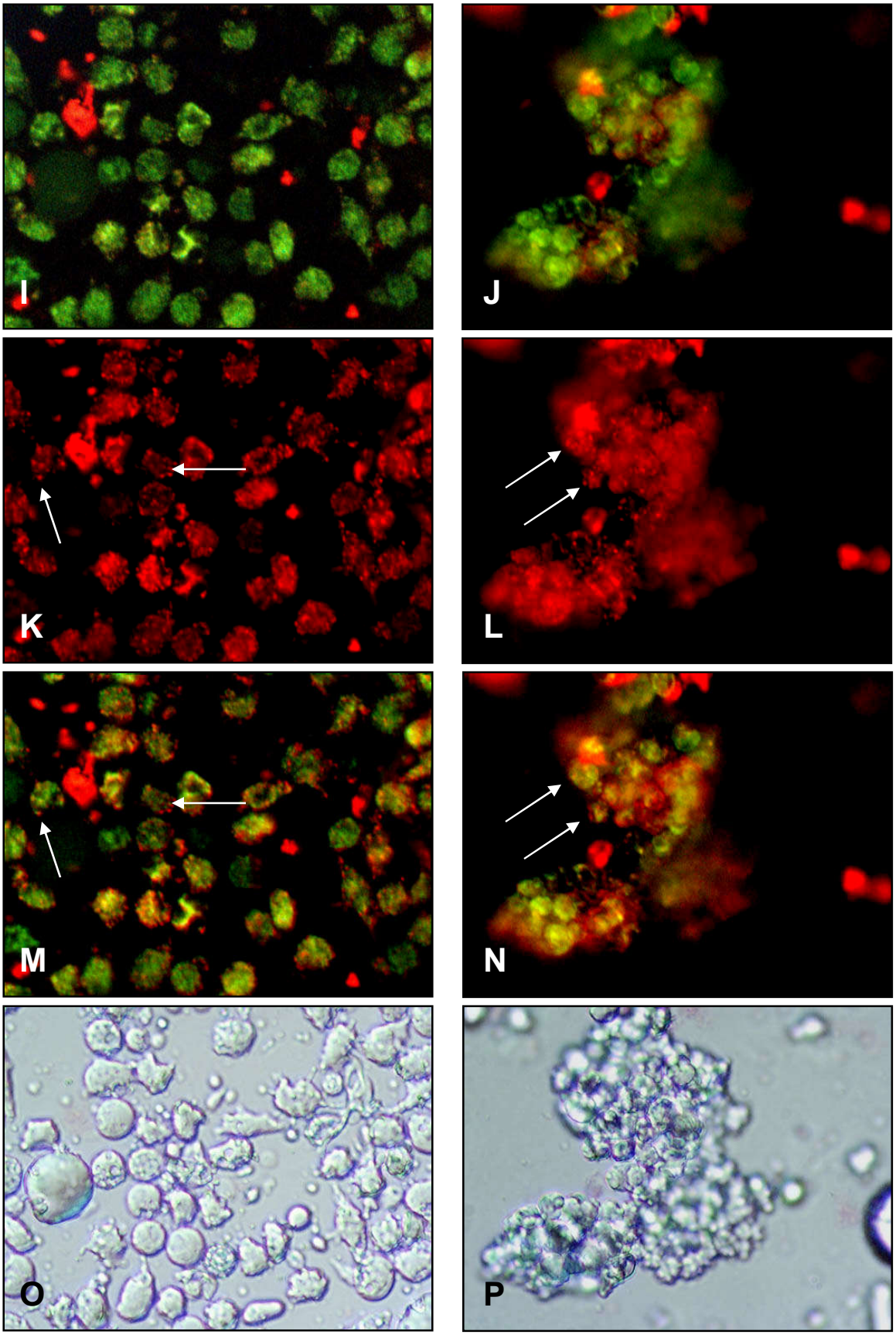

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Figure 10. Analysis of mitochondrial transmembrane potential in cycloheximide treated 64-cell stage blastomeres and germ-ring stage embryos. Embryos were collected and processed as is the materials and methods. A-H are 64-cell blastomeres, I-P are germ-ring stage embryos. A, C, E, G, I, K, M, and O are cycloheximide treated; B, D, F, H, J, L, N, and P are untreated controls. All magnifications are 200x. Each column is one set of cells. (A, B) 64-cell stage blastomere cells, image of monomeric form of DePsipher. (C) Cycloheximide treated 64-cell stage blastomeres lose the ability to form red DePsipher aggregates indicative of $\Delta\psi_m$ loss. (D) Untreated control blastomeres display extensive aggregate formation in mitochondria, (arrows). (E, F) Merged images of both forms of DePsipher. (G, H) DIC images of both treated and untreated forms of 64-cell stage blastomere animal pole cells. (I, J) Germ-ring stage animal pole cells image of monomeric form of DePsipher. (K) Cycloheximide treated germ-ring stage embryos display extensive amounts of red aggregates indicative of healthy $\Delta\psi_m$, (arrows). (L) Untreated control germ-ring stage animal pole cells display extensive aggregate formation (arrows). (M, N) Merged images of both forms of DePsipher. (O, P) DIC images of both treated and untreated forms of germ-ring stage animal pole cells.

Summary of Section II

Both 64-cell stage blastomeres and germ-ring stage embryos maintain plasma membrane integrity when treated with cycloheximide up to the hour before their animal pole cells rupture. When nuclear morphologies for 64-cell blastomeres and germ-ring stage embryos are compared after cycloheximide treatment, we see a very obvious apoptotic morphology in germ-ring stage embryos, but most of the cells in the 64-cell blastomeres display a necrotic morphology, with an occasional cell displaying chromatin condensation.

The TUNEL assay revealed that dying blastomere cells did not generate any DNA fragmentation that could be labeled by this assay. Germ-ring stage embryos treated with cycloheximide were heavily labeled by the TUNEL assay, revealing extensive DNA fragmentation. Agarose gel electrophoresis of cycloheximide treated germ-ring stage embryos revealed extensive internucleosomal DNA fragmentation. On the contrary, agarose analysis of 64-cell stage blastomeres revealed no visible signs of internucleosomal DNA fragmentation, results that were not surprising considering the data generated by the TUNEL assay.

The caspase-3 enzyme assays demonstrated that all early embryonic stages tested contained procaspase-3 and that they were all capable of processing the caspase-3 proenzyme into its active form. These data support recently published work that revealed the presence of procaspase-3 mRNA in

zebrafish early embryos (Yabu *et al.*, 2001b). 64-cell stage blastomeres lose their mitochondrial $\Delta\psi_m$ after 4 hrs when treated with cycloheximide, but germ-ring stage embryos retain their mitochondrial $\Delta\psi_m$ after equivalent treatment with cycloheximide. The loss of mitochondrial $\Delta\psi_m$ may be one of the reasons that 64-cell stage embryos display non-apoptotic nuclear features.

Results: Section III

Localization of Caspase-3 During Cycloheximide-induced Apoptosis in Germ-ring Stage Animal Pole Cells

In humans, the caspase family consists of 14 proteolytic enzymes (caspases 1-14). The enzymes have in common similar cleavage specificities, amino acid sequences, and are expressed as proenzymes. Different procaspases may be present at different intracellular compartments, and their localizations may change during apoptosis. In mouse liver, procaspases-3 is present in both cytosol and mitochondria. During Fas-induced apoptosis, caspase-3 is confined primarily to the cytosol (Chandler *et al.*, 1998). Caspase-3, the main effector caspase, is responsible for many of the morphological and biochemical cell changes that are the hallmark of apoptosis (Nicholson and Thornberry, 1997).

To determine the localization of caspase-3 during cycloheximide-induced apoptosis in zebrafish early embryonic cells a fluorometric live cell enzyme assay was performed. Embryos were incubated with the synthetic fluorogenic peptide substrate, Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC) (CALBIOCHEM, La Jolla, CA). The fluorometric live cell enzyme assay detects fluorescent emission of the molecule 7-amino-4-trifluoromethyl coumarin (AFC) after cleavage from the AFC-substrate conjugate, DEVD-AFC. Whole DEVD-AFC emits blue fluorescence (~400 nm) whereas after

proteolytic cleavage of the DEVD substrate by caspase-3 the freed AFC fluorophore emits a yellow-green fluorescence (~505 nm).

Active caspase-3 was localized to the cytoplasm of cycloheximide treated apoptotic germ-ring stage animal pole cells Fig. 11A. The intense staining displayed suggests that the active form of caspase-3 is found in extensive quantities throughout the cytoplasm. Untreated control embryos display no visible indications of caspase-3 activity Fig. 11B. These data suggest that procaspase-3 is present in a constitutive manner, and that processing of the zymogen causes its activation throughout the cytoplasm.

Figure 11. Caspase-3 live cell enzyme assay

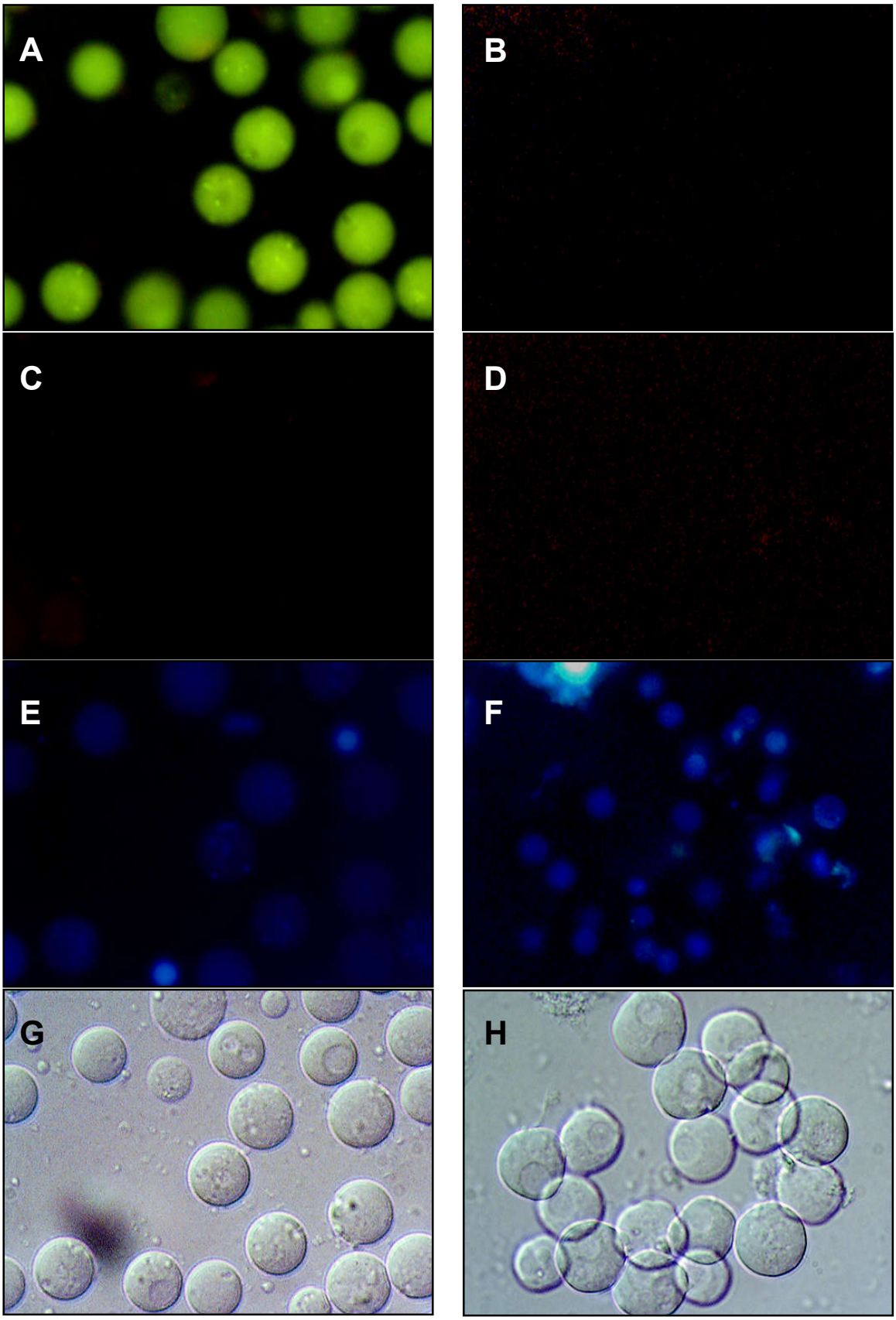


Figure 11. Caspase-3 live cell enzyme assay. Embryos were processed as in the materials and methods. All magnifications are 200x. A, C, E, G, and I are cycloheximide treated; B, D, F, H, and J are untreated controls. (A) Enhanced AFC fluorescence visualized in cycloheximide treated animal pole cells using an FITC filter, indicating an activation of the proenzyme form of caspase-3 in the cytoplasm, which in turn frees the AFC fluorophore from the AC-DEVD-AFC conjugate. (B) Untreated control zebrafish animal pole cells display no fluorescence, indicating caspase-3 inactivity under normal conditions. (C) Exclusion of propidium iodide from capase-3 positive animal pole cells reveals maintenance of cell membrane integrity in the earliest stages of apoptosis. (D) Exclusion of propidium iodide from control animal pole cells reveals normal maintenance of cell membrane integrity. (E) Chromatin condensation cannot be seen in the earliest stages of apoptosis as evidenced by the lack of chromatin condensation. (F) Normal nuclear morphology seen in untreated control animal pole cells. (G) DIC image of cycloheximide treated animal pole cells. (H) DIC image of untreated control animal pole cells.

Phosphatidylserine Localization in Cycloheximide Treated Germ-ring Stage Animal Pole Cells

Phosphatidylserine is a cell-membrane phospholipid found almost exclusively on the inner leaflet of the cell membrane. Phosphatidylserine translocation during apoptosis is an active process (Martin *et al.*, 1995). The translocation of this phospholipid to the outer leaflet suggests that during apoptosis it plays a key role in cell-cell signaling. Translocation of phosphatidylserine to the outer leaflet of the cell membrane is an early marker of caspase-3 activation during apoptosis (Martin *et al.*, 1995). Inhibition of caspase-3 activation by overexpression of Bcl-2 inhibits the translocation of phosphatidylserine to the outer leaflet of the cell membrane (Martin *et al.*, 1995).

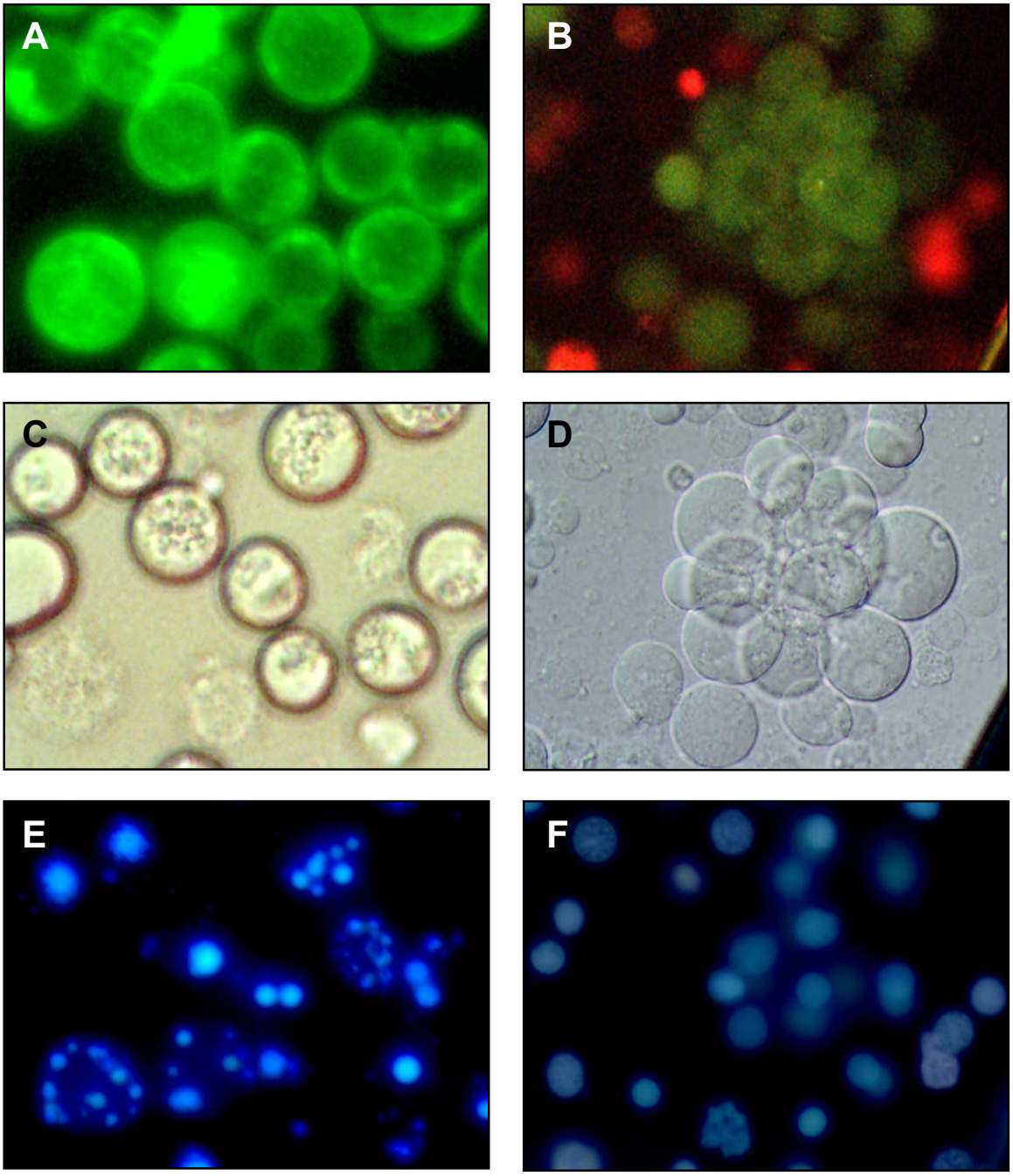
The human anticoagulant, annexin V, is a phospholipid-binding protein that has a high affinity for phosphatidylserine. To further analyze the role of caspase-3 in zebrafish early embryo apoptosis, phosphatidylserine localization was assayed with the Vybrant Apoptosis Assay Kit®, Alexa Fluor 488 conjugated to annexin V (Molecular Probes, Eugene, OR). Activation of caspase-3 by upstream initiator caspases leads to the cleavage of many cellular proteins, contributing to the orderly death of the cell.

Apoptotic and necrotic cells can be visualized in real time using fluorescence microscopy. Cells that have bound the annexin V /Alexa Fluor

488 conjugate will show intense green staining if they are apoptotic. If the cells have lost plasma membrane integrity, red staining from propidium iodide will be seen throughout the nucleus. Chromatin condensation seen with blue HOE staining and red PI staining co-localization is an indicator of secondary necrosis.

Cycloheximide treated germ-ring stage embryos translocate phosphatidylserine from the inner to the outer leaflet of the plasma membrane Fig. 12A. Untreated control embryos did not display any staining for phosphatidylserine Fig. 12B. This finding supports the belief that under normal conditions healthy cells position phosphatidylserine almost exclusively to the inner leaflet. These data indicate that zebrafish germ-ring stage embryos translocate phosphatidylserine possibly due to caspase-3 activation.

Figure 12. Detection of annexin V binding in apoptotic zebrafish early embryonic cells by fluorescence microscopy.



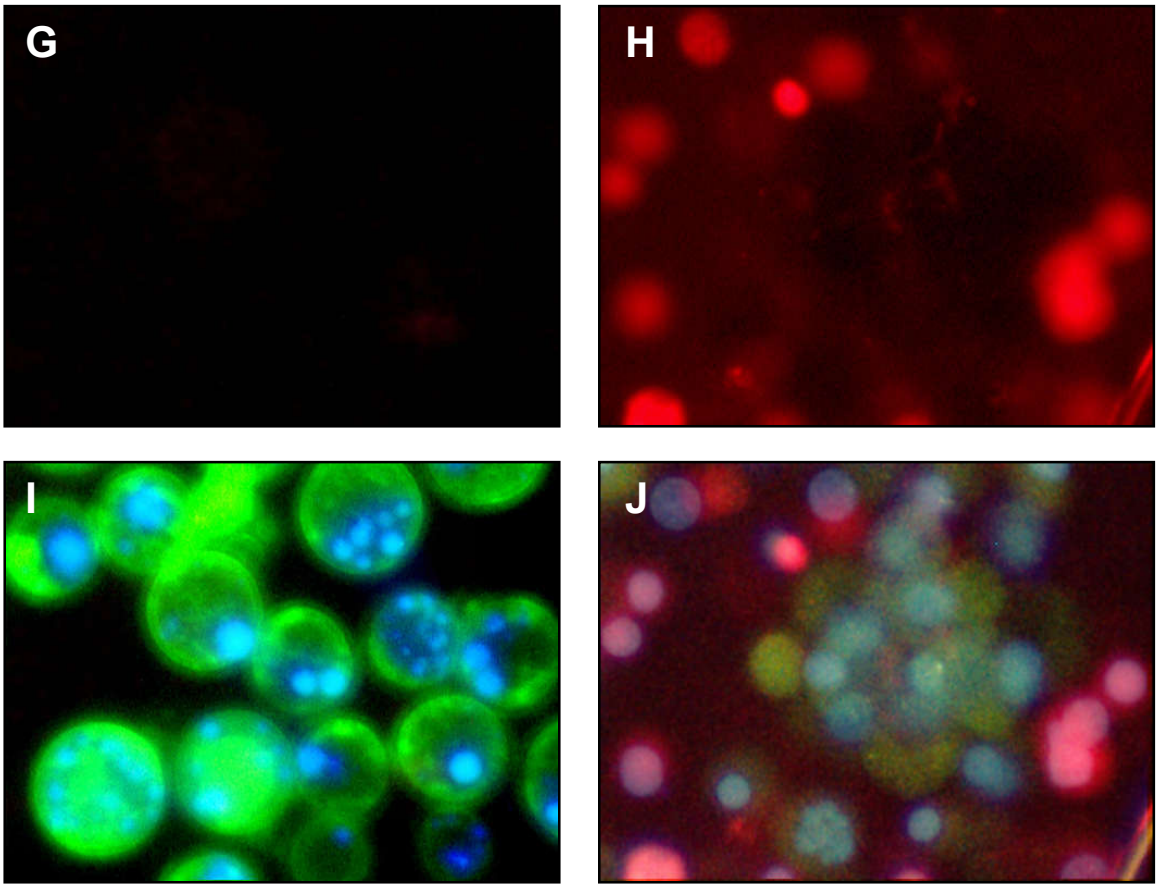

G
H
I
J

Figure 12. Detection of annexin V binding in apoptotic zebrafish early embryonic cells by fluorescence microscopy. Embryos were processed as in the materials and methods. All magnifications are 400x. A, C, E, G, and I are cycloheximide treated; B, D, F, H, and J are untreated controls. (A) Enhanced annexin V/Alexa Fluor 488 staining visualized in cycloheximide treated embryos using an FITC filter indicating a translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. (B) Untreated control zebrafish animal poles show weak staining of the cellular membrane, indicating normal cell membrane inner localization of phosphatidylserine. (C) DIC image of cycloheximide treated embryos. (D) DIC image of untreated control embryos. (E) Extensive chromatin condensation evident in cycloheximide treated embryos after 4 hrs perfusion. (F) Normal nuclear morphology seen in untreated control embryos. (G) Exclusion of propidium iodide from annexin V positive embryos reveals maintenance of cell and nuclear membrane integrity late into embryonic cell apoptosis. (H) Exclusion of propidium iodide from control embryonic cells reveals normal maintenance of cell membrane integrity. (I) Merged fluorescence images of cycloheximide treated embryos. (J) Merged fluorescence images of untreated control embryos.

Poly(ADP-ribose) polymerase (PARP) Cleavage During Germ-ring Stage Embryo Apoptosis

PARP is an enzyme that appears to be involved in DNA repair. Cleavage of PARP precludes the catalytic domain of PARP from being recruited to sites of DNA damage. At the onset of apoptosis caspase-3 cleaves PARP, inhibition of this cleavage attenuates apoptosis (Nicholson *et al.*, 1995). The active form of caspase-3 is capable of cleaving PARP into small molecular weight fragments during apoptosis (Lazebnik *et al.*, 1994). Mapping of the cleavage site of PARP identified the tetrapeptide DEVD as the consensus cleavage site for caspase-3 (Nicholson *et al.*, 1995). It is believed that cleavage of PARP during apoptosis ensures the completion and irreversibility of the process.

To confirm that cycloheximide-induced cell death in zebrafish early embryonic animal pole cells is apoptotic, western blot analysis for PARP was performed. The principle of the assay is the use of monoclonal Anti-PARP antibodies (CALBIOCHEM, La Jolla, CA) to detect both whole and cleaved PARP upon western transfer to a PVDF membrane and hybridization with primary and secondary antibodies. Both zebrafish PARP sequences, mRNA and protein, are unknown. When the sequences for other species of PARP were analyzed, the region most highly conserved was the region we chose to purchase antibodies for. We first tested whole recombinant bovine PARP

against our cytosolic extracts from cycloheximide treated embryos because we were not sure if the antibody was going to react with zebrafish PARP. After exposing bovine PARP to zebrafish caspase-3 active extracts, we were able to see multiple cleavage fragments by western blot. The expected fragment sizes were evident for caspase-3 cleavage of bovine PARP, but there were two extra bands seen on the western blot.

Upon further examination we realized that the reason there were extra bands was that the antibodies were also reacting with zebrafish PARP. We ran the assay again, but this time without the bovine PARP, and our results revealed that there were two bands that reacted with the antibodies. The first band was of a higher molecular weight and it was only found in extracts that were negative for caspase-3 with the fluorogenic enzyme assay. Extracts that were positive for caspase-3 activity displayed a second lower molecular weight band, the cleavage product caused by caspase-3. Control embryonic extracts should only display one band of PARP protein upon hybridization with the antibody. The activation of caspase-3 leads to the cleavage of PARP and the formation of a smaller molecular weight band in addition to the first whole PARP band. Both bands of PARP seen on the western blot are similar in size to PARP bands seen on western blots of apoptotic *Xenopus* embryos (Hensey and Gautier, 1997).

Cycloheximide treated germ-ring stage embryos display extensive PARP cleavage after the 5th hr of treatment Fig. 13. Cleavage of the protein is seen as the formation of low molecular weight fragments found to be approximately 25k daltons. Whole zebrafish PARP was approximately 70k daltons. These data suggest that caspase-3 activation results in the formation of PARP cleavage fragments, a hallmark of apoptosis.

Figure 13. PARP cleavage in germ-ring stage embryos exposed to cycloheximide.

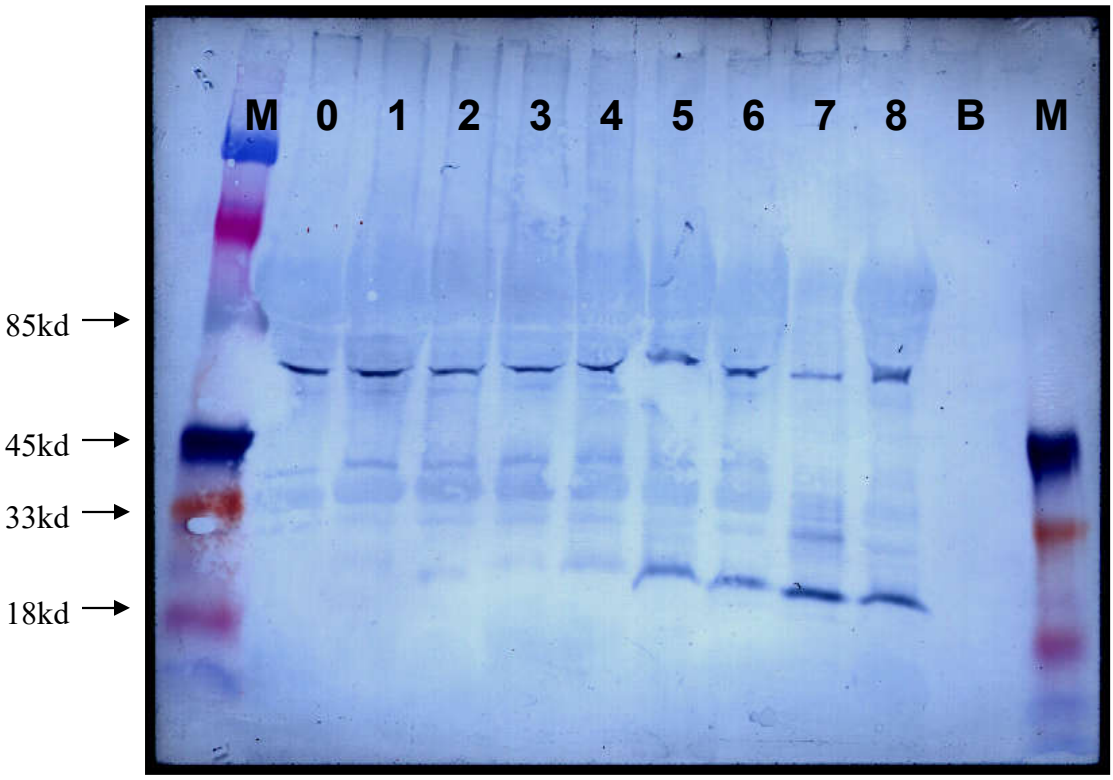


Figure 13. PARP cleavage in germ-ring stage embryos exposed to cycloheximide. Embryos were processed as in the materials and methods. Whole PARP can be seen in 0 hr untreated control zebrafish early embryos. Small amounts of cleaved of PARP can be seen after the 1st hr of cycloheximide treatment. Extensive cleavage of PARP is seen after the 4th hr of cycloheximide treatment, indicating that the embryo does not undergo extensive PARP cleavage until the 5th hr.

Transmission Electron Microscopy of the Nuclear Morphology Seen in Cycloheximide Treated Germ-ring Stage Embryos

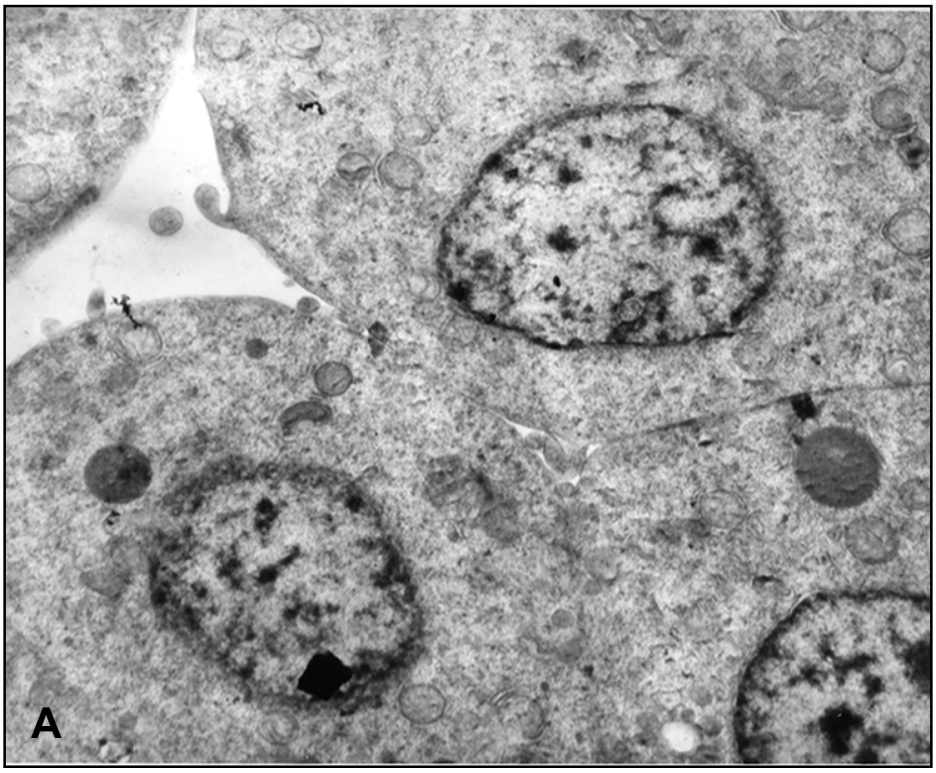
To confirm that cycloheximide treated zebrafish early embryos are dying in an apoptotic fashion, the nuclear morphology of dying embryonic cells was analyzed utilizing transmission electron microscopy. The identification of apoptosis is based primarily on the distinctive changes that take place within individual cells. Electron microscopy still provides the most reliable method for recognizing apoptotic morphology. The earliest and most obvious unequivocal morphological evidence of a classically apoptotic cell is found in the nucleus.

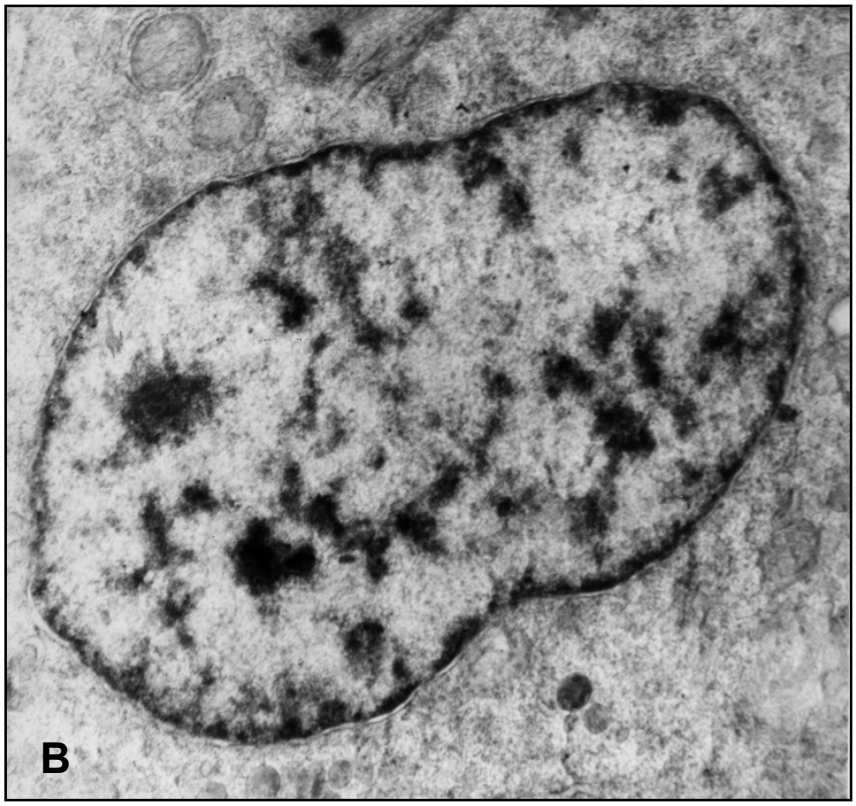
The first sign of apoptotic nuclear change begins with the condensation of nuclear chromatin into aggregates that migrate to the nuclear membrane. Slight convolution of the nuclear membrane outline is evident just before budding of the nucleus occurs. Condensed chromatin gathers at the convolutions, and as the convolutions become extreme, budding of the nucleus begins. The cytoplasm also undergoes condensation; this event has been associated with extensive protrusion or blebbing of the cell surface.

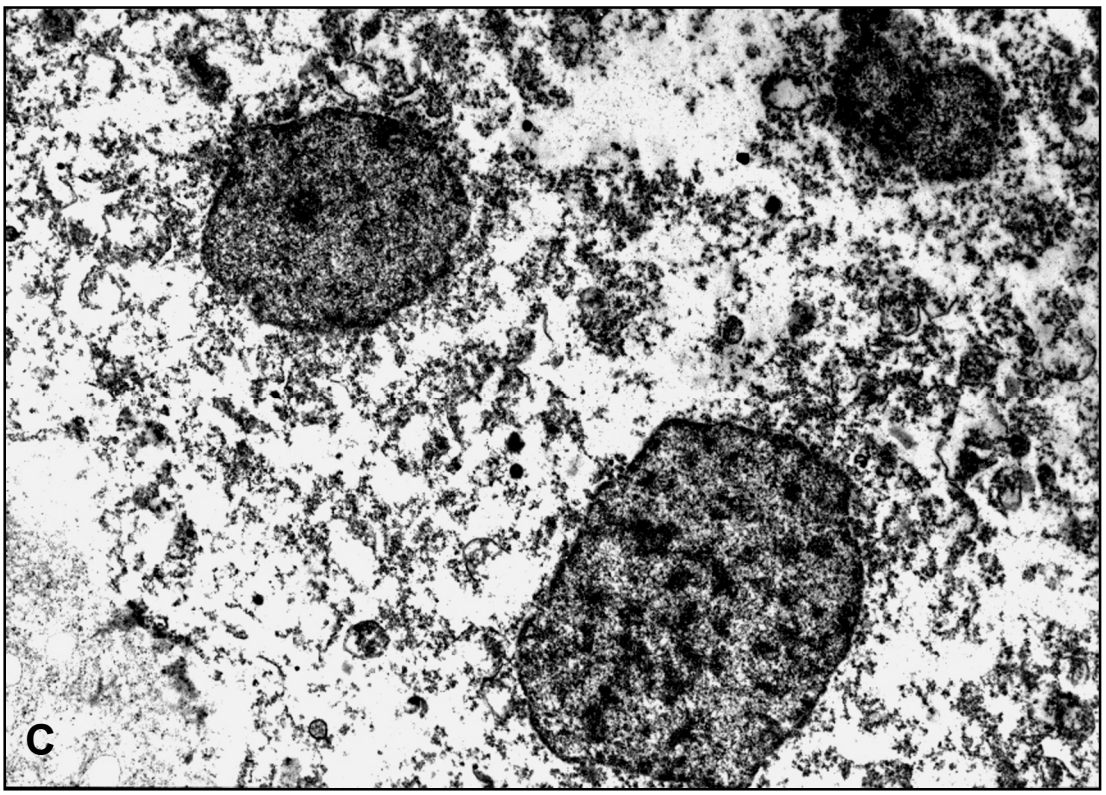
Caspase-3 activation is required for chromatin condensation and DNA fragmentation to occur during apoptosis (Woo *et al.*, 1998). Therefore, the classical nuclear morphology seen during apoptosis is dependent on the presence of functional caspase-3.

Germ-ring stage embryos display a classical apoptotic nucleus, complete with all the landmarks that accompany apoptotic elimination of the nucleus. After 2 hrs of cycloheximide treatment, germ-ring stage animal pole cells display nucleoplasm changes in chromatin structure (figure 14D). These changes can be seen as an initial condensation of the chromatin not seen in untreated control embryos. Cycloheximide exposure for 6 hrs produced extensive changes in nuclear morphology such as margination of condensed chromatin (figure 14F), convolution of the nuclear membrane (figure 14G), and the fragmentation of the nucleus (figure 14H). These micrographs provide us with undeniable evidence that germ-ring stage embryos treated with cycloheximide die an apoptotic death complete with all of the nuclear morphology changes associated with it.

Figure 14. Electron micrographs of apoptotic germ-ring stage embryo nuclei.



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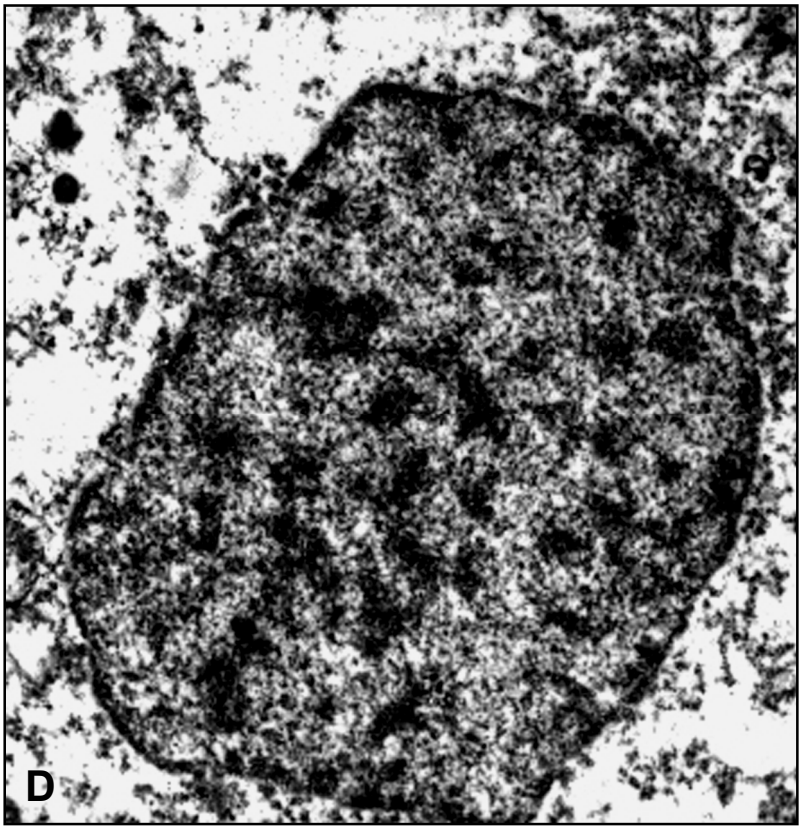
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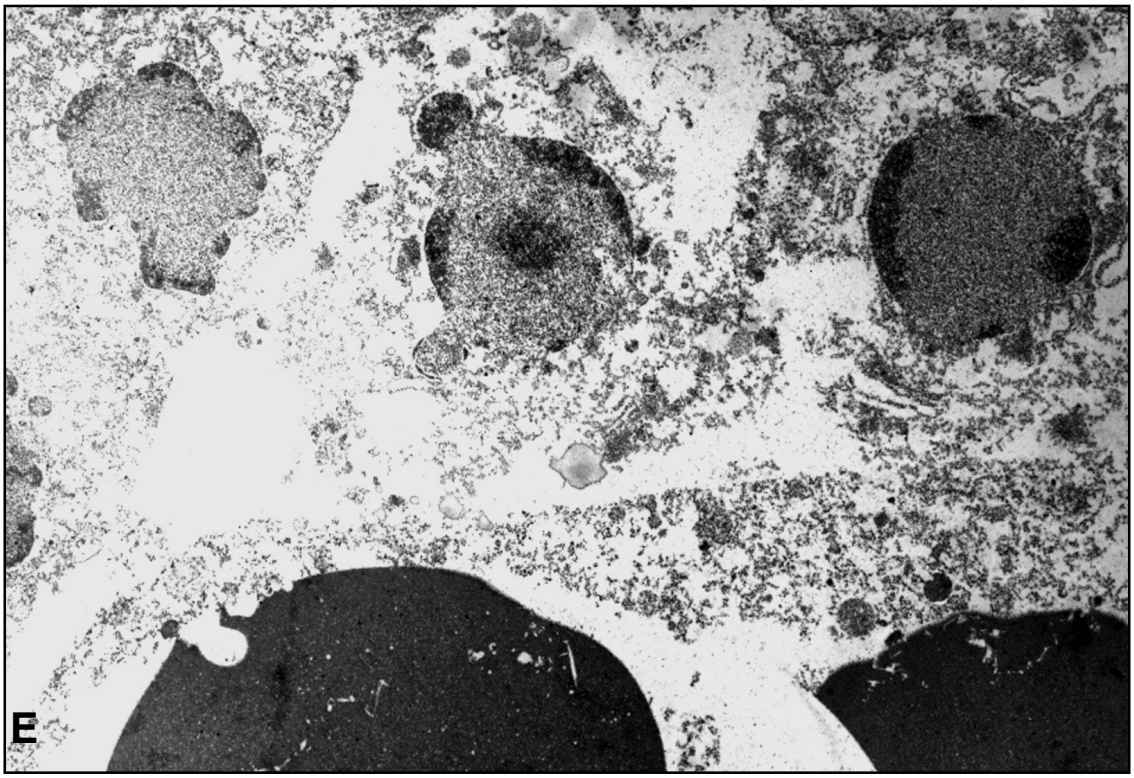
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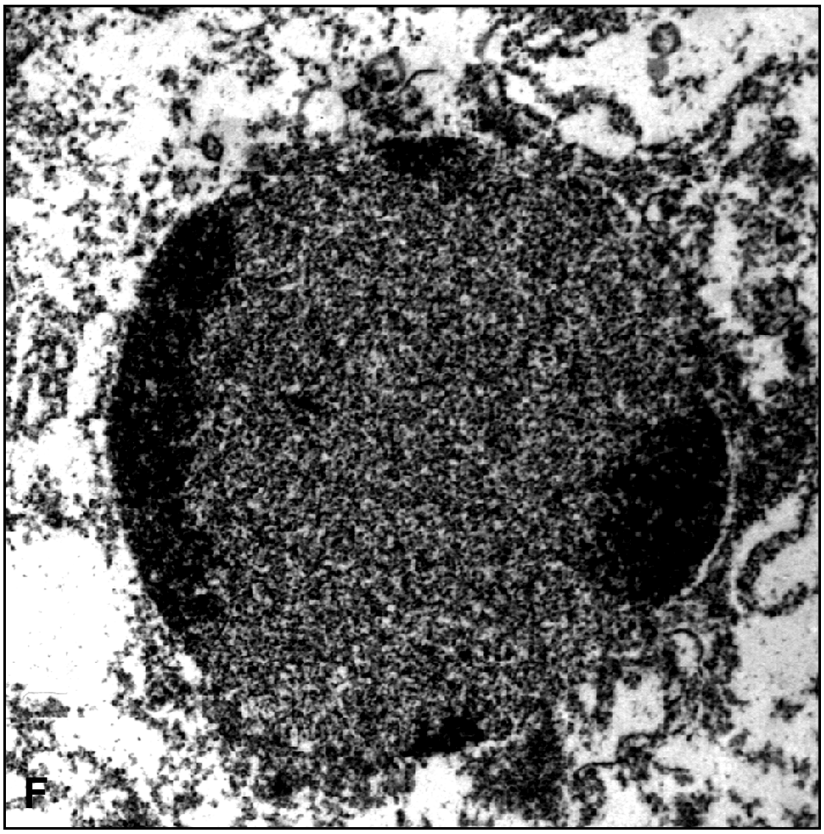
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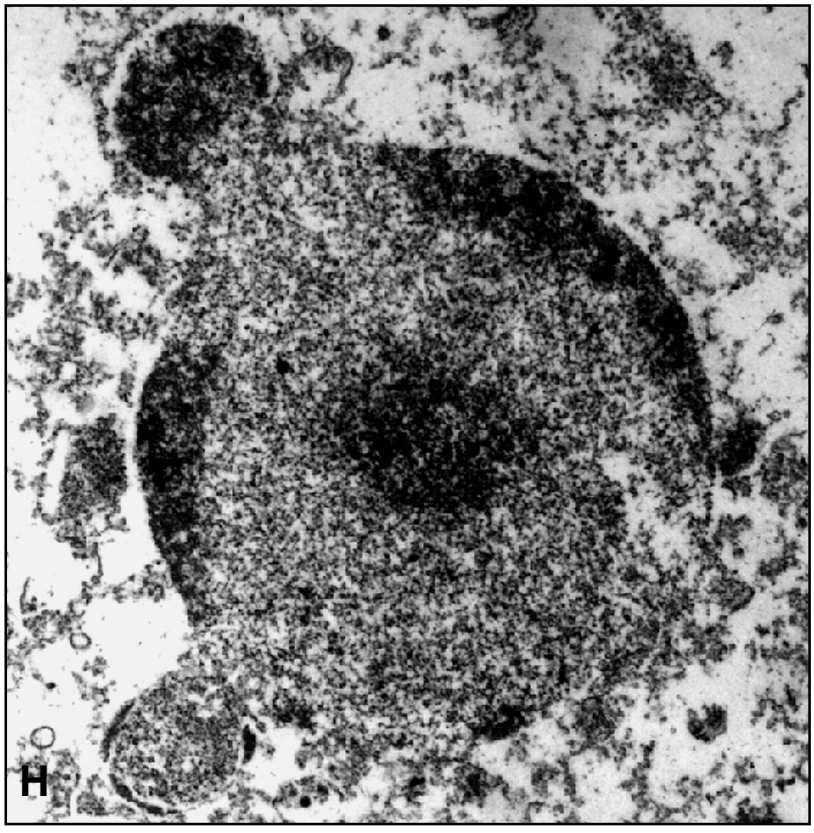
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Figure 14. Electron micrographs of apoptotic germ-ring stage embryo nuclei. Embryos were processed as in the materials and methods. Magnifications are as follows, A, C, and E are taken at 5000x; B, D, F, G, and H, are taken at 10,000x. A, and B are untreated controls, and C, D, E, F, G, and H are cycloheximide treated. C, and D were treated with cycloheximide for 2 hrs and E, F, G, and H were treated with cycloheximide for 6 hrs. (A) Untreated control embryos display scattered patches of chromatin in the nuclei of normally developing animal pole cells. (B) High magnification image of untreated control animal pole cell displays a clear nucleoplasm, an indicator of healthy nuclear chromatin. (C) Treated animal pole cells display a general increase in the chromatin condensation after 2 hrs cycloheximide. (D) High magnification image of a treated animal pole cell also displays an increase in the appearance of chromatin in the nucleoplasm after 2 hrs cycloheximide. (E) A classic apoptotic nuclear morphology can be seen in low magnification images after cycloheximide treatment for 6 hrs. (F) High magnification image of an apoptotic nucleus displays classic chromatin margination after cycloheximide treatment for 6 hrs. (G) High magnification image of an apoptotic nucleus displays extensive convolution of the nuclear membrane after cycloheximide treatment for 6 hrs. (H) High magnification image of an apoptotic nucleus displays extensive budding of the nucleus after cycloheximide treatment for 6 hrs.

Summary of Section III

Section III further examines cycloheximide-induced apoptosis in germ-ring stage embryos. The caspase-3 live cell assay revealed the presence of extensive caspase-3 activity in cycloheximide treated animal pole cells. Cycloheximide treated germ-ring stage embryos translocate phosphatidylserine to the outer leaflet of the cell membrane. The translocation of phosphatidylserine to the outer leaflet of the cell membrane has been shown to be linked to caspase-3 activation.

Cycloheximide treated germ-ring stage embryos cleave PARP, visible as a low molecular weight fragment on the immunoblot. PARP cleavage into specific molecular weight fragments is a marker of caspase-3 cleavage indicative of apoptosis. Germ-ring stage embryos also display extensive morphological changes in the nucleus when examined by transmission electron microscopy. Hallmarks such as chromatin condensation, nuclear membrane convolution, margination of chromatin, and nuclear fragmentation are clearly visible. All of these hallmark nuclear changes are apoptotic in origin.

CHAPTER IV

Discussion and Conclusions

Inhibition of Protein Synthesis and Survival Factor Deprivation

The induction of apoptosis through the use of protein synthesis inhibitors has been found to occur in many cell lines (Chang *et al.*, 1997; Hanusch *et al.*, 2000). The findings of these studies supported the argument that for some cell types interference with the cellular survival pathway leads to apoptosis. Induction of apoptosis by cycloheximide *in vivo* and *in vitro* is usually interpreted as the loss of an inhibitor or inhibitors of apoptosis (Collins *et al.*, 1991; Ledda-Columbano *et al.*, 1992; Martin *et al.*, 1990). In contrast, there have also been some instances where inhibition of protein synthesis does not stimulate apoptosis.

It was initially stated that B lymphocyte apoptosis does not require de novo protein synthesis because spontaneous apoptosis was increased by cycloheximide (Illera *et al.*, 1993; Norvell *et al.*, 1995). These findings favored the hypothesis of the pre-existence of the death machinery which must be restrained by the continuous synthesis of short-lived protective proteins, which include members of the Bcl-2 family (Illera *et al.*, 1993).

The consistent results seen in these studies served as the basis behind the use of cycloheximide for our experiments. All the cell death inducing

agents used so far in zebrafish have been targeted only at zygotic transcription and the synthesis of DNA. If the cell death machinery in zebrafish is constitutively expressed, then inhibition of protein synthesis should activate an already assembled cell death program. Microinjection of cycloheximide into cleavage stage zebrafish embryos caused a reduction in the levels of proliferating cell nuclear antigen (PCNA) after 10 to 25 min (Yarden and Geiger, 1996). The reduction in PCNA levels demonstrates the effectiveness of cycloheximide at inhibiting protein synthesis in zebrafish embryos.

Our original intent for the use of cycloheximide was to see if survival factors play a role in the control of caspase-3 activation. If the continual synthesis of survival factors is required by the embryo, then treatment with cycloheximide should inhibit this synthesis leading to an activation of apoptosis. The fact that zebrafish embryos arrest within the 1st hr of cycloheximide treatment suggests that cyclin synthesis has been disturbed; we interpreted this event as a general shutdown of protein synthesis (figure 2A1).

When cycloheximide is applied to 64-cell stage blastomeres, we get a substantial increase in caspase-3 activity by the 3rd hr of treatment (figure 9E). Because caspase-3 is known to be present as a proenzyme, its activation should require only activation of an upstream caspase. It is not know which caspase plays the initiator role in cycloheximide induced death but based on initial studies it can be inferred to be either caspase-8, or 9. When *Xenopus* embryos

were exposed to 100 μg/ml cycloheximide prior to the midblastula transition, all the embryos synchronously became apoptotic before the gastrula stage (Sible *et al.*, 1997). The use of emetine, another protein synthesis inhibitor, confirmed the results obtained with cycloheximide, that cycloheximide induced apoptosis occurred due to protein synthesis inhibition, and not from a particular toxicity of the drug (Sible *et al.*, 1997).

In *Xenopus*, the duration of the cycloheximide treatment is not what determines the time of death, as embryos exposed for 6, 8, and 10hrs all underwent cycloheximide-induced cell cycle arrest, and still all died synchronously at a time equivalent to stage 10.5 (Hensey and Gautier, 1997). When mouse eggs or 1-4-cell embryos were treated with 10μM staurosporine, with or without cycloheximide, for 26-60 hrs, however, most of the cells died by apoptosis as judged by their abnormal morphology, condensed chromatin, and TUNEL positive staining (Weil *et al.*, 1996). Thus, it seems that mouse blastomeres and eggs seem to be capable of undergoing apoptosis and do not need to initiate *de novo* protein synthesis to do so. The same could be said of the zebrafish 64-cell stage blastomere and germ-ring stage embryo.

Programmed Cell Death in Zebrafish Eggs

The roots of the cell death program found in zebrafish early embryos is of maternal origin. This program's ability to work is first demonstrated before the formation of the zygote. We have demonstrated that if left unfertilized for an extended period of time the zebrafish egg will undergo apoptosis in caspase-3 dependent fashion. Enzyme analysis for caspase-3 activity reveals that it is relatively inactive for the first 4 hrs of culture, but at the 5th hr caspase-3 activity markedly increases, and over the next 3 hrs increasing 3 fold by the time of death 8 hrs post-activation (figure 9D).

The fact the fertilized zebrafish eggs eventually form zygotes and develop normally to adulthood in the same culture medium and conditions proves that the death of the egg is not because of unsatisfactory culture conditions, but because of the lack of fertilization. It seems that this program is set up at the formation of the oocyte and activated when the egg is water activated in culture causing the block to polyspermy. This constitutive presence of cell death machinery in the egg is a classic example of how cell death is highly regulated from conception, or the lack of it, to adulthood.

In higher vertebrates, if the ovum is not properly fertilized, death of the ovum eventually occurs. In humans, it is known that the first day of ovulation is the most fertile day of a female's cycle whereas insemination on the day following ovulation rarely results in a successful pregnancy (Wilcox *et al.*, 1995).

Thus it seems that an ovulated human egg ages rapidly, losing the ability to give rise to a vital conceptus within 24 hr of ovulation. It has previously been demonstrated that unfertilized eggs and polar bodies are eliminated through apoptosis (Takase *et al.*, 1995; Warner *et al.*, 1998a). Thus, it seems that the ovum is predestined to die unless fertilization and embryogenesis are successful. The same conclusion can be drawn for the zebrafish egg given the data we have accumulated.

The starfish egg contains a similar default pathway for unfertilized eggs. Unfertilized starfish eggs arrest in postmeiotic interphase, and they eventually die if left unfertilized (Yuce and Sadler, 2001). Postmeiotic interphase arrested starfish eggs persist for 16-20 hrs, then the eggs synchronously and rapidly die (Yuce and Sadler, 2001). The dying eggs extrude membrane blebs, fragment their cytoplasm, and condense their DNA (Yuce and Sadler, 2001). Treatment of starfish eggs with the caspase-3-specific inhibitor Ac-DEVD-CHO, blocked membrane blebbing and fragmentation in eggs treated with 1-methyladenine (Sasaki and Kazuyoshi, 2001). These results demonstrated the importance of caspase-3 in the morphology and biochemistry of apoptosis in starfish eggs.

Future attempts at blocking caspase-3 activation in zebrafish should shed some light on the importance of caspase-3 for egg cell death. If the unfertilized zebrafish egg relies solely on caspase-3 as its death effector, then inhibition of its activity may prolong the lifespan of the egg. Whether it remains viable

enough to give rise to an embryo if sperm is injected into the cytoplasm is another story.

Programmed Cell Death in Zebrafish 64-cell Stage Blastomeres

64-cell stage blastomeres undergo rapid cell death when treated with cycloheximide (figure 4C). Analysis of plasma membrane integrity revealed that the blastomeres maintained their plasma membrane integrity up to the 4th hr of culture, as evident by the exclusion of PI (figure 5D). But by the 5th hr most of the cells in the animal pole rupture, probably due to the cells' inability to maintain osmotic homeostasis. When the nuclear morphology is analyzed, we do not see any changes in the way AO stains the DNA in the nucleus (figure 5C).

64-cell stage blastomeres treated with cycloheximide rapidly activated caspase-3 throughout the blastomere. Yet the morphological hallmarks associated with caspase-3 activation are rarely found in the dying blastomeres, suggesting that there was an initial activation of the apoptosis machinery, but only of the earlier stages due to the lack of middle and late stage apoptosis landmarks. There is a lack of classically condensed nuclei in the majority of the dying cells in the 64-cell stage blastomere, yet some isolated instances of chromatin condensation can be seen (figure 6D and E).

Agarose gel electrophoresis analysis of the genomic DNA from 64-cell stage blastomeres revealed that no internucleosomal DNA fragmentation was present, even though caspase-3 was activated (figure 8B). The lack of a ladder pattern suggests that caspase-3 either cannot cleave the zebrafish equivalent of

ICAD or that the abrupt death of the embryo occurs before fragmentation of the DNA can occur. TUNEL analysis of dying 64-cell stage blastomeres confirmed that none of the cell in the animal pole generated fragmentation of their genomic DNA (figure 7C).

Caspase-3 activation is seen in as little as 2 hrs in 64-cell stage blastomeres treated with cycloheximide (figure 9E). It can be inferred from this evidence that the pathway inhibiting caspase-3 activation is lost by the 2nd hr of cycloheximide treatment. This activation occurs much earlier than most previously reported hallmarks of an apoptotic cell death. Activation of caspase-3 may be due to the loss of inhibitory factors required for cell survival. The loss of protein synthesis is believed to be the driving factor for the loss of the survival pathway. The rapid activation of caspase activity suggests that protein synthesis is required in 64-cell stage blastomeres to inhibit the activation of caspase-3. This is the first data that has demonstrated this necessity this early in zebrafish development.

Analysis of mitochondrial $\Delta\psi_m$ reveals that there is a total loss in the ability to produce ATP by the 4th hour of treatment in 64-cell stage blastomeres treated with cycloheximide, as displayed by the DePsipher assay (figure 10C). This loss in the ability to produce ATP could be the reason why blastomeres lose the ability to get past the earlier stages of apoptosis. Wherever ATP levels

have been directly measured in apoptotic cells, no rapid drop has been observed (Ankarcrona *et al.*, 1995; Shimizu *et al.*, 1996a). Given that the apoptotic process is highly dependent on ATP for its activity (Ankarcrona *et al.*, 1995), there is a possibility that energy depletion may be to blame for the lack of apoptotic morphology.

Neonatal rat cardiac myocytes treated with staurosporine under ATP-depleting conditions were found to be primarily necrotic. However, under ATP-replenishing conditions, staurosporine induced an apoptotic death in increasing number of cells (Shiraishi *et al.*, 2001). This evidence suggests that the presence of ATP favors a shift from necrosis to apoptosis through caspase activation. In human T cells that were predepleted of ATP, staurosporine-induced cell death lacked any visible signs of nuclear condensation and DNA fragmentation (Leist *et al.*, 1997). Repletion of ATP prevented necrosis and restored the ability of the cells to undergo apoptosis (Leist *et al.*, 1997). This outcome suggests that ATP was required for the active execution of the final phase of apoptosis, which involves nuclear condensation and DNA degradation.

Programmed Cell Death in Zebrafish Germ-ring Stage Embryos

Germ-ring stage zebrafish embryos displayed many of the characteristic changes associated with apoptosis after cycloheximide treatment. Under low magnification, the first visible sign of apoptosis that was seen was a loss of cell-cell adhesion in the animal pole (figure 3A2). The process of cell shedding is interpreted as the loss of cell adhesion proteins from the cell surface. This phenomenon can be found in most models of apoptosis, from *C. elegans* to *H. sapiens*. One of the requirements for the fragmentation of a cell is that it must detach itself from its neighboring cells. Once detachment occurs, bleb formation ensues, leading to the fragmentation of the cell.

Analysis of plasma membrane integrity after chronic cycloheximide treatment revealed that animal pole cells from germ-ring stage embryos retain their plasma membrane integrity late in the cell death process. Maintenance of plasma membrane integrity during the later stages of death is a hallmark of apoptosis. Our results from the AO/PI Live-Dead assay clearly show that even though the nuclei of the animal pole cells contain highly condensed chromatin, stained by AO, plasma membrane integrity is still intact, as revealed by the exclusion of PI from the cytosol, and nucleus (figures 5G and H).

One of the most visible signs that a cell has become apoptotic is the condensation of its chromatin into clumps. Cycloheximide treated germ-ring stage embryos display many of the characteristic nuclear morphological

changes associated with apoptosis. Extensive nucleoplasmic chromatin condensation was seen by the 2nd hr post-cycloheximide treatment (figure 14C and D). Chromatin clumping, convolution of the nuclear membrane, margination of the condensed chromatin, and the fragmentation of the nucleus eventually ensued, all of which were clearly visible in apoptotic animal pole cells (figure 14E, F, G, and H).

The extent to which genomic DNA has undergone fragmentation can most effectively be demonstrated by agarose gel electrophoresis. Internucleosomal DNA fragmentation results from extensive cleavage of genomic DNA by CAD, which is activated as the result of caspase-3 activation. The genomic DNA of germ-ring stage embryos displayed extensive internucleosomal DNA fragmentation after just 2 hrs of cycloheximide treatment (figure 8A and C). This outcome reveals that the germ-ring stage embryo can reach the later stages of apoptosis within 2 hrs.

We believe that most of the initial internucleosomal DNA fragmentation seen in this assay originated from the cells of the enveloping epithelial monolayer (EVL), but as time progressed cells from the deep layer contributed to the intense amount of fragmentation seen on the gel. Zebrafish mid-gastrula stage embryos treated with nocodazole produced DNA fragmentation in all three cell layers of the embryo (Ikegami *et al.*, 1997b). TUNEL labeling of cycloheximide treated germ-ring stage embryos confirmed that the genomic

DNA was extensively fragmented (figure 7A). This assay detected the generation of 3'-OH strand breaks, a hallmark of apoptotic DNA degradation.

Recently published work on zebrafish caspase-3 was the first to analyze the presence of its transcripts during early embryogenesis (Yabu *et al.*, 2001b). The transcripts were detected as early as the formation of the zygote, and throughout all the early embryonic stages. Functional conservation of the cysteine protease is not known. Our lab focused on caspase-3 at the biochemical level, looking for signs of its function in situ, in cytosolic extracts, and through the use of Annexin V and immunoblot analysis for PARP cleavage.

In situ analysis of caspase-3 activity revealed that activated caspase-3 is present throughout the cytoplasm in cycloheximide treated germ-ring stage embryos. A live cell assay for active caspase-3 revealed that zebrafish caspase-3 was capable of cleaving the Ac-DEVD-AFC substrate in live cells (figure 11A). Cytosolic extracts from cycloheximide treated germ-ring stage embryos were assayed for caspase-3 activity, showing that zebrafish caspase-3 was capable of cleaving the Ac-DEVD-AFC substrate after 2 hrs of treatment (figure 9F).

One of the earliest signs of caspase-3 activation is the translocation of phosphatidylserine from the inner to the outer leaflet of the cell membrane (Martin *et al.*, 1995). Cycloheximide treated germ-ring stage embryos successfully translocate phosphatidylserine to the outer leaflet of the cell

membrane (figure 12A). This is the first report of this occurrence in zebrafish embryos. The role of phosphatidylserine translocation has not been studied in the zebrafish, but its translocation during apoptosis suggests that it plays a role in bleb uptake by neighboring cells and macrophages. Because phosphatidylserine translocation is seen only when caspase-3 is activated, this suggests that the mechanism for its translocation is conserved in zebrafish.

PARP cleavage is a conserved hallmark of apoptosis. Its cleavage during zebrafish apoptosis has not been studied. Cycloheximide treated germ-ring stage embryos displayed extensive cleavage of PARP after 4 hrs of treatment (figure 13). The importance of PARP cleavage during apoptosis in mammals was revealed when the insertion of an uncleavable PARP (lacking the caspase-3 cleavage site) into MEF cells causing a delay in cytoplasmic blebbing and nuclear disassembly during apoptosis (Oliver *et al.*, 1998).

The Maternal-Zygotic Transition and the Midblastula Transition

The zebrafish egg is primed for death once it is laid. Our caspase-3 data indicate that the lack of fertilization causes this death protease to be activated. If fertilization occurs, the embryo develops normally, indicating that there must be some type of cell death inhibition that occurs during the formation of the zygote. We believe that this inhibition of apoptosis is effective against insults with inhibitors of transcription and replication as shown by our colleagues (Ikegami *et al.,* 1997a, and b; Ikegami *et al*., 1999), yet our data suggest that this inhibition of apoptosis is lost when an inhibitor of translation is used.

The maternal-zygotic transition (MZT) is characterized by depletion and degradation of maternal determinants (mRNA and protein) and their replacement by zygotic mRNA and protein. The MZT begins during the blastula period and persists until the end of gastrulation and into segmentation. Fertilization of the egg enables the zygote to develop unimpeded to mid-gastrulation. Zebrafish 4 and 8-cell blastomeres treated with aphidicolin and hydroxyurea remained alive for an extended period of time until they died at 8-10 hrs of age (Ikegami *et al*., 1999). If the same treatments are applied to embryos between the 16 cell stage and the MBT, most of the embryos survive past gastrulation stage in control embryos (Ikegami *et al*., 1999). In 3 hpf embryos treated with UV irradiation at 20 mJ/cm^2, caspase-3 activity increased beginning at 8 hrs post-irradiation, with the activity eventually increasing 7.5

times that of normal embryos after 12 hrs (Yabu *et al.*, 2001a). When 3 hpf embryos were treated by heat shock and irradiation, TUNEL-positive cells were observed 8 hrs post-treatment (Yabu *et al.*, 2001a). Pre-MBT treatment with the microtubule destabilizer nocodazole produces complete destruction of all nuclei in the deep cell layer of zebrafish embryos after the MBT (Ikegami *et al.*, 1997b).

We used 64-cell stage blastomeres for our experiments because of their position on the timeline of development. This stage occurs 2 hpf, one hour before the beginning of the MZT/MBT. When 64-cell stage blastomeres are exposed to cycloheximide, they arrest at a stage equivalent to a pre-MBT blastomere, so morphologically they never progress into the MBT. At the biochemical level, they should also have pre-MBT proteins. Zygotic transcription should not occur because of the stunting of the embryo's development. We forced the 64-cell blastomere to activate caspase-3 during a time at which activation of the protease should be inhibited. This outcome suggests that the MZT/MBT is not as important in cycloheximide induced apoptosis as it is in other contexts. Whether caspase-3 has been activated in the context of the other zebrafish pre-MBT embryos is unknown. Our use of cycloheximide on 64-cell stage blastomeres displayed a different response when compared to the other systems. Much of the death was delayed until a later period in the blastomeres exposed to an inhibitor of transcription and a

microtubule destabilizer, but blastomeres treated with cycloheximide activated caspase-3 rapidly and died much quicker.

Treatment of *Xenopus* 2-cell stage embryos with α-amanitin, an inhibitor of transcription, had no effect on the early cell divisions, whereas treatment with the protein synthesis inhibitor cycloheximide caused an arrest in cell division within 1 hr of treatment, due to the inhibition of cyclin synthesis (Hensey and Gautier, 1997). When the 4-cell arrested embryo was exposed to cycloheximide for 7.5 hrs, equivalent to stage 9, TUNEL staining was still not detected (Hensey and Gautier, 1997). When cycloheximide was applied continuously at concentrations that block 97% of protein synthesis, *Xenopus* embryos arrested cell division but did not die until a time when they would normally be gastrulating, approximately stage 10.5, which is the time when they become TUNEL positive (Hensey and Gautier, 1997).

Cycloheximide pulsed *Xenopus* embryos examined prior to the midblastula transition contained chromatin that appeared damaged, without apoptotic bodies, suggesting that apoptosis does not occur prior to the midblastula transition (Sible *et al.*, 1997). These data were similar to our data generated with the zebrafish 64-cell blastomeres with the exception that we saw extensive caspase-3 activation, something the other authors did not examine.

This begs the question, what defines apoptosis? Is it the activation of caspase-3 or the morphology associated with its activation?

The evidence gathered leads us to believe that an inhibition of apoptosis in pre-MZT/MBT zebrafish early embryo is setup at the formation of the egg, and that this inhibition is probably upstream of procaspase-3. Our data suggest that this inhibition relies on the continual synthesis of survival factors that can be disrupted with the use of cycloheximide. Our data support the possible existence of cell death clock which is present at the formation of the egg and persists in fertilized and unfertilized embryos until a time equivalent to 8 hpf in control embryos. The existence of a clock-like mechanism is supported by the fact that populations of water-activated eggs die synchronously by the 8th hr of culture.

Support for our argument can be found in work done by Kane and Kimmel, 1993. If unfertilized eggs are activated by simply exposing them to water, they undergo a very long series of rapidly occurring cyclic changes characteristic of cell cycles during cleavage. The eggs never undergo the extensive cycle lengthening seen in normal embryos at cycle 10 through 12. For the first 15 cell cycles, the cycle period is close to that of normal cleavage. They describe it like a clock winding down, the period progressively slows down to 30 min, by the time when the eggs lyse, at about 8 hrs post-activation (Kane and Kimmel, 1993). Thus, in water-activated eggs, cell cycle-like

oscillations occur at 15 minute intervals, and do so long after the normal time of cell cycle lengthening. This same clock is probably responsible for the activation of the cell death machinery found in the unfertilized egg.

We think that the definition of apoptosis must be further examined to differentiate what requirements must be met to label a dying cell apoptotic. If caspase-3 activation defines apoptosis, then we see apoptosis in our 64-cell stage blastomeres. If the morphology associated with caspase-3 activation is what defines the death, then our 64-cell stage blastomeres could be considered to be undergoing a type of secondary necrosis.

Conclusions

In conclusion, we have demonstrated that functional cell death machinery can be found in the zebrafish early embryo before zygote formation. During oocyte formation maternal factors, mRNA and proteins, are supplied to the oocyte in large quantities. Within this pool of factors is the machinery required for programmed cell death, which has been shown to be functional by the cytoplasmic extract assay for caspase-3 activity. This evidence suggests that the egg is constitutively programmed to die, and if it is left unfertilized for a prolonged period of time, a self-destruct command is given.

Analysis of 64-cell stage blastomere cell death reveals a general lack of apoptotic nuclear morphology during death, yet we see activation of capase-3. The morphological hallmarks associated with caspase-3 activation are rarely found in the dying blastomeres, suggesting that there was an initial activation of the apoptosis machinery, but only of the earlier stages due to the lack of middle and late stage apoptosis landmarks. Analysis of mitochondrial $\Delta\psi_m$ reveals that there is a total loss in the ability to produce ATP in 64-cell stage blastomeres treated with cycloheximide as displayed by the DePsipher assay. This loss in the ability to produce ATP could be the reason why blastomeres lose the ability to get past the earlier stages of apoptosis. Given that the apoptotic process is

highly dependent on ATP for its activity, there is a possibility that energy depletion may be to blame for the lack of apoptotic morphology.

Caspase-3 activity and the hallmarks associated with its activation can be seen in germ-ring stage embryos treated with cycloheximide. Fluorometric enzyme analysis for caspase-3 like activity revealed a sharp increase in the presence of active caspase-3 in cytosolic extracts incubated with the fluorogenic caspase-3 substrate Ac-DEVD-AFC after 4 hrs CHX exposure. Western blot analysis revealed that PARP was cleaved by caspase-3, evident by the presence of low molecular weight PARP fragments after 4 hrs CHX exposure. *In situ* enzyme analysis using Ac-DEVD-AFC revealed that active caspase-3 was found throughout the cytoplasm during the earlier stages of apoptosis. Cytoplasmic caspase-3 activity was seen before any morphological changes to the nucleus were seen. Annexin V staining confirmed that there was a translocation of phosphatidylserine to the outer leaflet of the cell membrane in animal pole cells during the earliest stages of apoptosis, before any morphological landmarks could be seen.

Our data suggest that the use of cycloheximide to inhibit protein synthesis effectively activates caspase-3 in a yet unknown manner. This activation seems to be evident throughout the early embryonic period, from the 64-cell stage blastomere through to the germ-ring stage. The ability of cycloheximide to activate caspase-3 before the MZT/MBT suggests that the

inhibition seen in our colleague's research is regulated at the translational level. In their system there is no inhibition of translation. The use of cycloheximide in our system reveals that there exists a delicate survival factor balance that can be overcome within 2 hrs of treatment with cycloheximide, evident by the activation of caspase-3.

Whether there is an inhibition of apoptosis before the MZT/MBT in zebrafish early embryos can not be answered by our use of cycloheximide due to the activation of caspase-3 that we see. But given the work that has been done in zebrafish with an inhibitor of transcription, and a microtubule destabilizer, and the delay of death that they see in their research it suggests that there is some sort of inhibition.

Based on our research on unfertilized eggs, we conclude that if the egg is left unfertilized for a prolonged period of time, then an apoptotic response is elicited at a time corresponding to midgastrulation. It may be that the zebrafish egg inherits inhibitors of apoptosis from its mother during the formation of the egg, and that these inhibitors are maintained by the maternal mRNA that is also inherited. Our use of cycloheximide two hours after fertilization supports this argument because we see caspase-3 activation at a time when we know protein synthesis can not occur; therefore, the maternal inhibition has been silenced. The fact that it takes 8 hrs for the unfertilized egg to die suggests that the egg may only contain enough mRNA to inhibit apoptosis for that amount of time.

By this time during normal embryogenesis the fertilized egg will have already developed into midgastrulation, and transcription and translation will be controlled by the zygote. So this phenomenon could be compared to the changing of the guard. If the zygote is unable to sustain survival factor levels by the time the maternal inhibitors are depleted, then the death of the embryo will ensue.

Based on this research, it can be inferred that many of the morphological and biochemical features of caspase-3 dependent apoptosis seen in higher vertebrates are conserved in zebrafish, and that the maternal factors inherited during oocyte formation play a key role in the regulation of the programmed cell death machinery during zebrafish early embryogenesis.

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