

Apoptosis in amphibian development

Jean-Marie Exbrayat, Elara N. Moudilou, Lucie Abrouk, Claire Brun

Université de Lyon, UMRS 449, Biologie Générale, Université Catholique de Lyon, Reproduction et Développement Comparé, Ecole Pratique des Hautes Etudes, Lyon, France

Email: jmexbrayat@univ-catholyon.fr

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ABSTRACT

Amphibians and more particularly *X. laevis* are models often used for studying apoptosis during embryonic development. Using several methods, researchers determined the localization of programmed cell deaths (PCD). Several experimental methods also have been used to understand the regulatory mechanisms of apoptosis, throughout development, contributing to elucidate the general action of several genes and proteins. Apoptosis occurs very early, with a first program under control of maternal genes expressed before MBT, in order to eliminate damaged cells before gastrulation, and a second program at the onset of gastrulation. PCD is also observed during neurulation. Then, apoptotic cells are observed in amphibian organogenesis and metamorphosis. Results of these researches showed both importance of PCD for embryonic development, and the complexity of its regulation. Results obtained can be useful to understand others aspects of the importance of apoptosis, particularly pathological aspects.

Keywords: PCD; Apoptosis; Development; Metamorphosis; Amphibian; *Xenopus*

1. INTRODUCTION

Amphibians reproduce in water with an external or internal development at the end of which the tadpole or larva brings on metamorphosis, turning in a terrestrial adult. Apoptosis being a major event on metamorphosis, amphibians, and more especially *Xenopus laevis* are useful models to study programmed cell death [1-4]. A synthesis of works about apoptosis, obtained from amphibians is given here.

2. GENERAL ASPECTS OF EMBRYONIC DEVELOPMENT IN AMPHIBIANS

Eggs and early embryos of amphibians are characterized with the presence of a pigmentation which clearly indicates a bilateral symmetry. Fertilized eggs immediately

divide to give the first two blastomeres which continue to divide becoming a morula. An inner cavity, the blastocoel, appears in the mass cell of the embryo, becoming a blastula. During this period of cleavage, the size and shape of embryos do not vary. Before mid blastula transition (MBT), zygotic genes do not express excepted those encoding for proteins implicated in membrane building. Maternal mRNAs previously accumulated in the oocytes remain present in the cytoplasm of zygote and they are distributed into the cytoplasm of blastomeres during cleavage. These maternal mRNAs encode for proteins which will be implicated in expression of zygotic genes. In post-MBT, zygotic genes express according to maternal signals. After cleavage, cells displace during gastrulation, at the end of which ectoderm, mesoderm and endoderm are installed. The archenteron or primitive intestine is observed. After gastrulation, on the dorsal part of the embryo, the neurectoderm originates successively the neural plate, then neural folds which fuse to form the neural tube. The dorsal part of mesoderm differentiates onto notochord and somites. After neurulation, the end of which being characterized by "tail-bud" stage, organogenesis occurs. After a growth period, metamorphosis occurs. This phase corresponds to the transfer from an aquatic to a terrestrial life and it is characterized by the modification, development, or regression of several organs.

3. APOPTOSIS AT EARLY STAGES: SEGMENTATION AND GASTRULATION

3.1. Repartition of Apoptotic Cells during Normal Development

Repartition of apoptotic cells in early stages of development has been little described [5-7] but consistent data have been published in developing *X. laevis* [8]. At the beginning of development, first TUNEL positive cells appear in embryos at the onset of gastrulation. Staining was first observed such as spots or symmetric masses in the region surrounding the blastopore. Apoptotic cells were also found in the remaining blastocoel. Histological

investigations showed the presence of apoptotic cells on ectoderm and internal mesoderm. At the end of gastrulation, apoptotic cells described a dorsal median strip or two parallel strips on the dorsal part of embryo, showing the place of future neural plate. TUNEL positive cells were found in both neuroectoderm and underlining mesoderm. Degenerative cells were also observed in the Japanese newt *Cynops pyrrhogaster* [7]. At the onset of gastrulation, strongly pigmented cells were observed in ectoderm and in the blastocoel. In old gastrulae, cells showing a similar aspect were found in ectoderm, on contact with archenteron. At the end of gastrulation, two groups of pigmented cells were localized into the residual blastocoel and in the anterior part of archenteron. Dead cells in *Cynops pyrrhogaster* and *X. laevis* showed a similar repartition. In developing zebrafish *Danio rerio* dead cells also presented a corresponding repartition [9, 10], showing this phenomenon of cell death at gastrulation is general in lower vertebrates.

3.2. Regulation of Apoptosis during Segmentation and Gastrulation

To understand the regulation of apoptosis, experimentations have been performed on early development stages in *X. laevis* [11-13]. After irradiation with γ rays damaging DNA before the first cleavage, embryo continued to cleave. The 12 first cycles of divisions were perfectly comparable to that of control animals with additional divisions. Irradiated DNA being shorter than normal one, its replication was faster, explaining the presence of additional divisions. So, the time of segmentation used to attempt gastrulation was genetically regulated by a timer. In embryos DNA of which being broken with restriction endonucleases before the first division, synchronization of cleavages was not perturbed and time to attempt gastrulation was not modified. So, embryonic DNA was not implicated in control of these cell cycles.

In two cells-embryos treated with α anamitine in order to inhibit transcription, segmentation was no effected. In embryos treated with cycloheximide, an inhibitor of protein synthesis, cell division was stopped one hour after the treatment. In embryos treated with a mixture of aphidicoline and hydroxyurea used to block replication of DNA, the segmentation was also stopped. All these treatments consequently provoked a rapid and synchronous death of embryos at the onset of gastrulation with cells showing apoptotic characteristics. In addition, a caspase activity was shown in extracts of irradiated embryos at stage 10.5, contrarily in non-irradiated cleaving embryos or gastrulae.

Two programs of cell death from segmentation to tadpole stage have been observed [12]. One of these programs was activated at the onset of gastrulation, conse-

quent to the degradation of cells occurring before MBT. It was supposed that the degradation of these non stable cells was programmed by maternal genes, according to a biological timer independent on the type of stress, cell progression, or protein synthesis. A second program of cell death started at gastrulation in normal embryos. In embryos of *X. laevis* treated with hydroxyurea, an instability of cyclins A1 and A2 was observed at the onset of gastrulation [14]. At this period, zygotic transcription was necessary to suppress apoptosis in normal embryos. In treated animals, it was not possible to maintain such an inhibition and consequently a lot of apoptotic cells were observed. So, the apoptotic program was suppressed in early blastulae by maternal inhibitor, and the degradation of mRNAs encoding for apoptosis inhibitors before MBT, was then compensated with a new zygotic transcription between MBT and gastrulation. In other experiments, apoptosis was blocked with injection of mRNAs encoding for BCL-2. Results confirmed that maternal program was blocked with inhibitor of apoptosis. In animal submitted to low doses of cycloheximide, development was delayed before MBT but DNA synthesis, and transcription started normally at the onset of gastrulation. All these results suggested the existence of a checkpoint at MBT, regulated by maternal genes in order to start apoptosis and also showed that zygotic transcription could block maternal program of apoptosis [15].

The lengthening of cell cycle from MBT was due to an increase of phosphorylation of Tyr16 belonging to cdc2. Resistance to apoptosis was associated to an increase of p27 Xie1, a part of BCL-2 or Bax-2 implicated in pro- or anti-apoptotic complexes, and an increase of protein kinase Akt. At MBT, the degradation of cyclin E was also regulated by an internal timer insensible to the inhibition of DNA, mRNAs and proteins [16], provoking apoptosis at gastrulation [6]. An injection of cyclin A2 suspension did not affected early development and the embryos were able to attempt tail-bud stage if cdk2 was added. After MBT, an injection of cyclin A2/cdk2 characteristic of G1/S phases provoked a cell proliferation, more especially in the future epidermis disturbing the equilibrium between division and differentiation.

In *X. laevis*, the maternal program of apoptosis was activated just after MBT with an over-expression of S adenosyl methionine carboxylase [17], provoking a lack of S-adenosyl methionine. Embryos developed in blastula, but stopped at the onset of gastrulation at which cells became apoptotic. This destruction was stopped with an injection of BCL-2 mRNA. In embryos receiving micro injection of 5-aza-CdR inducing hypomethylation of DNA, or 5-methyl dCTA, inducing contrarily a hypermethylation, cleavage was normal but embryos became apoptotic at gastrulation [18]. Apoptosis was suppressed in embryos injected with an inhibitor of caspases

and the activation of caspase-9 was a stage-key for the activation of maternal program [19]. XChk1 provoked an elongation of cell cycle [20]. If Chk1 was inhibited, the development was normal until the onset of gastrulation at which the embryo died, with cells entering in apoptosis. In zygotes with two blastomeres injected with *SAMD1* mRNAs, apoptosis was also observed at gastrulation [21, 22]. If these mRNAs were injected on a side only in embryos with 4 to 8 cells, a large number of animals became tadpoles; if embryos were 16 to 32 cells, all individuals became tadpoles with sometimes abnormalities. Apoptotic cells originating from injected blastomeres were found in blastocoel, even in developing embryos. Other molecules have been signaled in order to participate to the check-point of apoptosis [23]. High levels of p27^{BBP}/eIF6 have been observed during development when PCD increased. This anti-apoptotic factor was related to an increase of apoptosis in the parts of embryos needing cell death for harmonious development [24]. Several experiments based upon the over-expression of *FoxO* genes in *X. laevis* showed these genes were indispensable for tissue differentiation but not for gastrulation, even overexpression of several of them induced severe damages in gastrulae [25]. *Bix* expressed in early *Xenopus* gastrula, and an over-expression as well as a depletion of *Bix3* causes apoptosis [26].

In other vertebrates, embryos also exhibit the activation of surveillance mechanisms, early in development, to produce the selective apoptosis of damaged cells like in amphibians. [27].

The interactions between embryonic layers were studied in order to understand regulation of balance between cell survival and apoptosis [28]. Glucocorticoid-inducible kinase 1 (SGK1) promoted ectodermal cell survival during early *Xenopus* embryogenesis. A dorsal depletion of SGK1 resulted in modified morphology. In transgenic embryos, a knockdown of SGK1 caused apoptosis in the ectoderm. SGK1 also stimulated production of BMP7. Finally the existence of chain of reactions with an endodermal and mesodermal pathway from PI3K to SGK1 to the transcription nuclear factor producing BMP7 was demonstrated, promoting ectodermal survival by decreasing Death Inducing Signaling Complex function. In *Xenopus*, maternal p53 mRNAs and proteins seem to be essential for development [29].

4. APOPTOSIS AT EARLY STAGES: NEURULATION

4.1. Description of Apoptosis in Normal Development

The presence of apoptotic cells was detected during neurulation of *X. laevis* [8]. At stage 13, apoptotic cells visualized were localized on the dorsal part of embryo,

generally distributed on two strips or a single median strip, delimitating the neural plate from blastopore to the dorsal part of body. Apoptotic cells were localized in the neuroectoderme and the underlining mesoderm. At stages 14 and 15, TUNEL labeled cells were grouped on the anterior part of neural plate. At stages 16 and 17, apoptotic cells were particularly numerous on both the anterior and dorsal parts of embryos, corresponding to nervous system. The most intense TUNEL staining corresponded to the presence of primary sensorial neurons. Staining was particularly intense in the region of future brain, at the place of olfactive and otic placodes. Comparable data have been obtained in the zebrafish *Danio rerio* [9]. At the end of the stage of neural plate, the number of apoptosis decreased comparatively to previous stages. In some embryos, apoptosis were also visualized in the notochord [30], and they became more and more numerous till the end of neurulation, with an anterior-posterior pattern of distribution. If apoptosis was inhibited in mesodermal explants of notochord, this one became twice longer than in control, but in some embryos, notochord lacked a recognizable structure, apoptotic cells were observed in the tail but somites were not affected. Apoptotic cells appeared in neural crests. From stage 26 to 28, apoptotic cells were distributed in two symmetric areas in brain, and also observed in eyes, spinal cord and tail. Cell deaths were also observed on neural tube in two neurulae of *Cynops pyrrhogaster*, with a distribution like in *X. laevis* [6].

4.2. Regulation of Apoptosis at Neurulation

During neurulation, several primary neurons visualized with N tubulin became apoptotic, like in sensorial placodes in which apoptosis were observed prior the formation of synapse. In *X. laevis*, this part of embryo was under the expression of the homeobox gene *Xotx2* but, at the beginning of neurulation, the expression of *Xrx1* occurred and expression of *Xotx2* stopped. The first definition of the territories of retina on diencephalon in early neurulae resulted from the repression of *Xotx2* by *Xrx1*. So, *Xrx1* could be implicated on properties of the anterior part of neural plate in order to develop the anterior brain and eyes [31]. In *X. laevis* embryos in which cell proliferation was blocked, the spatio-temporal pattern of apoptosis remained unaffected in neuroectoderm if they contained half the normal number of cells. An over-expression of *Bcl-2* during primary neurogenesis of *Xenopus* embryos stopped PCD. After inhibition, the normal neurogenesis was disrupted with perturbation of expression domains of several genes. Yet, inhibition of PCD did not affect the outcome of lateral inhibition. These experimentations demonstrated that PCD regulated primary neurogenesis at the level of neuronal determination [32].

In *Xenopus POSH* (Plenty of SH3s) was implicated in the development of anterior brain, and essential for apoptosis when it was mediated by c-Jun N-terminal kinase (JNK) [33]. The importance of expression of gene *XBtg2* was demonstrated in *Xenopus* neural development [34]. The expression of this gene was found on the anterior part of neural plate and the neural crests at mid-neurula stage. If *XBtg* was inhibited on only one side of embryo, formation of eyes became impaired, and the anterior neural development disrupted. In embryos in which *XBtg2* was depleted, a decrease in the expression of neural genes was observed in anterior brain, but neither in neural crests nor in epidermis, suggesting *XBtg2* implicated for the differentiation of the anterior neural plate in which its depletion provoked an increase of both cell apoptosis and proliferation.

In *X. laevis*, *msx1* gene acted as a factor promoting apoptosis, and *Slug* gene acted as an anti apoptotic factor [35-39]. Both the anti-apoptotic gene *Bcl-2* and the apoptosis-promoting gene *Bax* disturbed the expression of these genes. A *Slug* over-expression provoked an enlargement of the neural crests and derivatives. Region of neural crests in which *Slug* was expressed, did not undergo apoptosis, and region in which *msx1* was expressed became apoptotic in the part of neural folds adjacent to the neural crest. The balance between these two antagonist genes controls the formation of neural crest cells and derivatives. Prohibitin 1, an inhibitor of cell proliferation, presented a function in development of neural crests. Its gene *Xphb1* was maternally and zygotically expressed. In neurula transcripts, it accumulated in the neural tube and the neural crests [40]. Inhibition of *Prohibitin1* resulted in the loss of expressions for several genes whereas other genes were not affected. E2F1-binding domain was necessary for the action of *Xphb1* in development of neural crests [40].

The repartition of p27BBP/eIF6, a protein the decrease of which was accompanied by a reduction in proliferation of several cells in eIF6 mice, was studied throughout the development of *Xenopus* [41]. At the beginning of embryogenesis, a correspondence between highest levels of p27BBP/eIF6, cell proliferation and apoptosis was observed. In later development stages high proliferation levels were present in the same regions where higher p27BBP/eIF6 expression was observed, while apoptosis were not concentrated in the same sites. So, a high presence of p27BBP/eIF6 would appear in the regions where cell death is essential for normal development [41].

5. APOPTOSIS DURING ORGANOGENESIS AND GROWTH PERIOD

Between stages 35 and 39, a high number of apoptotic

cells were observed in the whole organism, but the organs essential for a free life did not present any dead cells. Dead cells were observed especially in ventral part of the prosencephalon and in spinal cord. The retina presented two phases of cell death [42], one at stages 37 - 38 in the ventronasal part of retina, and the other at stage 47 in retinal ganglion cell layer [43]. At stage 46, when the hind limbs appeared [2], TUNEL positive cells were mainly observed in the epidermis and the first generation of gills. Few apoptosis were then observed in the second generation of gills. During the period at which the cement gland, a transitory organ, began to degenerate, nervous system and several organs presented numerous dead cells [44]. At these stages, tadpoles were very sensitive to environmental changes and became fragile [1, 43]. During the end of the growth period up to stage 52, no massive cell death occurred.

6. APOPTOSIS AT METAMORPHOSIS

6.1. Several Aspects of Apoptosis at Metamorphosis

Amphibian metamorphosis involves dramatic remodeling of organs, so a large quantity of apoptosis occurs permitting the replacement of larval organs and tissues with the adult ones. In *X. laevis*, metamorphosis is three days long, and the end is characterized by the entire regression of the tail [45-51]. Amphibian metamorphosis is a good model to understand the mechanisms regulating PCD [52-54]. The tail regression implicates an apoptotic pathway inducible by T3 hormone and involving some cell death executioners [55].

At the onset of metamorphosis, numerous apoptotic cells were observed in the spinal cord and caudal spinal ganglia [43,47,56], and a caspase-3 activity increased during climax in the spinal cord [48]. At stage 58, a peak of apoptosis was observed in spinal cord [43,56]. The number of limb motoneurons decreased strongly after limb innervations [57-59]. From stage 61, cell death in the spinal cord was increasingly observed affecting motoneurons and ependymal cells. PCD in the motoneurons started behind the onset of PCD involving sensory neurons [43,56]). At stage 64, some nervous cells were still observed in the residue of the tail [60]. The elimination of several retinal ganglia cells occurred during climax [61,62]. It was supposed that the process of optic nerve remodeling accompanied the displacement of eyes from lateral to more dorsal and rostral position as the frog acquired binocular vision studied [62,63]. The competition for target sites might also modulate retinal PCD [64]. Cell deaths were also observed in some brain regions such as mesencephalon and diencephalon. The number of apoptotic cells in the brain increased at the onset of metamorphosis. At stage 61 numerous apoptotic

cells were observed in the thalamus and mesencephalon. The primary sensory Rohon-Beard neurons [65] and the giant Mauthner's neurons in the hindbrain regressed during metamorphosis, between stages 50 to 55 [66,67]. The expression of the *xR11* gene prolonged the survival of the Rohon-Beard cells and limited the morphological changes of Mauthner's cells [67,68] but did not affect the disappearance of motoneurons of spinal cord.

At climax, the muscles of the tail totally regressed [10, 45]. At stage 57, muscle cells began to be altered [40,51] and fast muscular fibres were deleted first [59]. At stage 62, TUNEL positive cells of muscle were detached from the extracellular matrix and died [60] and finally caudal muscles were removed [53,69]. Cell death occurred also in the dorsal muscles which do not express the adult contractile proteins, in order to be progressively replaced with adult muscle [70]. Caspase-3 activity and *Bax* mRNA [71] increased in muscle cells during the climax, suggesting that death of tail muscle cells implicates a caspase-dependent pathway. *Bax* gene is regulated during metamorphosis [72]. The regression of tail implicates an apoptotic pathway inducible by T3 hormone [58]. *Caspase 9* mRNA was expressed in the tail before metamorphosis and increased before and during climax, the production of active forms of caspase 9 increased in muscle tissue as metamorphosis progressed [73].

At metamorphosis, the cells of larval intestine became apoptotic, and they were replaced with non-differentiated cells in order to form adult intestine [74-76]. *TRβ* participated to apoptosis of larval intestine; close relationship between the epithelium and the connective tissue was evident during the intestinal remodeling [77-79]. The occurrence of apoptosis was also demonstrated in the different parts of digestive tract during metamorphic remodeling in *Rana pipiens* and *Ceratophrys ornata* larvae [80]. Matrix metalloproteinase MMP-9TH was responsible of apoptosis in the larval epithelial through degrading ECM components in the basal lamina, whereas MMP-9 was involved in the removal of dying epithelial cells during intestinal remodeling [81]. Digestive tract was remodeled under the control of epithelial-connective tissue interactions. In larval epithelial, cells underwent apoptosis, while a small number of stem cells proliferated and differentiated to form adult epithelium [82]. In liver, gene expression changed drastically but no major morphological changes were observed [76].

In the skin, the outer two-cell layers of the epidermis died by apoptosis during the climax [59], showing the presence of caspase-3 [83]. At metamorphosis, number of larval red blood cells decreased whereas adult ones increased. During metamorphosis, many larval red blood cells expressed TUNEL-positive reactions in the spleen [84].

6.2. Regulation of Metamorphosis and Apoptosis

Thyroid hormones, T3 and T4 start amphibian metamorphosis [51]. T3 and T4 concentrations present a peak at stages 58 to 66. Thyroid hormones regulated genes expression through its nuclear receptors (TRs). In *Xenopus*, *TRβ* genes were highly expressed only during metamorphosis [85]. *Xenopus TRβ* genes were regulated in a cell-type specific manner [86], and implicated in both inducing apoptosis and stimulating cell proliferation. TRs could form heterodimers with retinoic receptor binding with the thyroid hormone response element situated in the target gene promoter which they active in presence of thyroid hormone during metamorphosis, so thyroid hormone receptors and 9-cis retinoic acid receptors were required for mediating the regulation of the genes [49,87,88]. Many genes were up-and down-regulated by thyroid hormone in the regressing tail [89]. TH also played a direct role in neurogenesis during development of connections between the spinal cord and limbs.

The developmental expression of thyroid hormone response genes, encoding extracellular matrix-degrading metalloproteinases, suggested that extra cellular remodeling plays an important role, including cell death, cell proliferation and differentiation. Since some years, studies concerning the role of extra-cellular matrix have been published, showing its importance on metamorphosis [87, 90-93]. The expressions of *MT1-MMP* and *GelA* genes were studied in intestine and tail of *X. laevis* during metamorphosis [92]. Both genes were up-regulated when both organs underwent metamorphosis. *MT1-MMP* only was also expressed in the longitudinal muscle cells of the metamorphosing intestine. *MT1-MMP* and *GelA* function together in the extracellular matrix degradation or remodeling with independent roles in muscle development in the intestine. At the onset of metamorphosis, the expression of lamin LA was down-regulated, but that of lamin LIII was up-regulated only in the islets of progenitor cells [94]. Then, the expression of LA became up-regulated, whereas that of LIII became down-regulated in the adult cells. Results suggested the involvement of the lamins in the process of dedifferentiation during amphibian metamorphosis.

Concentration of intracellular calcium is one of the factors that can modulate apoptosis. So, an excessive influx of extracellular calcium can activate enzymes which participate to cell death. Consequently, an activation of ionotropic receptors like AMPA/kainate receptors permeable to calcium may participate to the elevation of intracellular calcium. In *X. laevis*, calpains, auxiliaries of signal transduction for apoptosis [95], could be implicated during events of metamorphosis when activated by calcium [43]. In order to understand these complex

mechanisms, in a first time, units GluR2/3 of AMPA/Kainate receptors were in several organs of *X. laevis* tadpoles in stages 50 and 54 [96], other organs remaining negative. In central nervous system, periventricular radial cells, motoneurons and ganglia cells were also visualized. Then, apoptotic cells were visualized in *X. laevis* embryos from hatching to climax [56]. Several organs always contained apoptotic cells. Transitory organs were affected by apoptosis only at regression period. Nervous cells degenerate, with a peak of apoptosis at stage 58, just before tail regression. The effect of NBQX, an antagonist of AMPA/kainate receptors of glutamate on PCD was researched in tadpole of *X. laevis* at stage 46 [97,98]. The ubiquity of calpains 1, 2, 3, was observed in all the organs, with a weak expression at early stages, increasing in tadpoles and decreasing at metamorphosis [99]. In NBQX treated animals, the number of apoptotic cells increased variably in the organs, with a stronger label in those particularly affected by metamorphosis [97,98].

7. CONCLUSION

Amphibians, with more particularly *X. laevis* and *X. tropicalis* [100], but also other species [6,80,101] are useful models for studying apoptosis during embryonic development. For that, it was first necessary to give a table of development. So, the normal table of *X. laevis* [2] is a useful tool. In order to understand apoptosis during normal development, searchers determined first the localization of cell deaths. Then, several experiments permitted to understand the mechanisms of apoptosis throughout development, contributing to elucidate the general action of several genes and proteins in its regulation. Apoptosis occurs very early, with a first program under control of maternal genes used to eliminate damaged cells before gastrulation, and a second program at the onset of gastrulation. PCD was also observed during neurulation. In mice, the role of apoptosis was studied in the regulation of forebrain development [102]. In mice in which genes *Jnk1* and *Jnk2* have been deleted, no apoptotic cells were observed at stage of neural fold, and the posterior part of hindbrain remained open in advanced embryos; in mice for which *caspase-3* was deficient, the cerebral wall thickened with a convoluted area of cortex in surviving embryos. Then, PCDs were observed in amphibian organogenesis and metamorphosis. Results of these researches showed both the complexity of PCD and its importance for embryonic development. They can also be useful to understand others aspects of the importance of apoptosis, particularly in pathologies.

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