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Control of Programmed Cell Death During Zebrafish Embryonic Development

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Abstract

Programmed cell death (PCD) is a conserved cellular process, which is essential during embryonic development, morphogenesis and tissue homeostasis. PCD participates in the elimination of unwanted or potentially harmful cells, and contributes in this way to the precise shaping of the developing embryo. In this review, the current knowledge related to the role of PCD during zebrafish development is described and an overview is provided about the main actors that induce, control and execute the apoptotic pathways during zebrafish development. Finally, we point out some important issues regarding the regulation of apoptosis during the early stages of zebrafish development.

Keywords: Bcl-2 family, apoptosis, cell death, embryonic development, mitochondria

1. Introduction

What would today be called genuine apoptotic cells were first observed by German scientist Carl Vogt in 1842. He was studying the morphogenesis of the tadpole notochord of the midwife toad *Alytes obstetricans* when he observed the formation and subsequent disappearance of vesicular nuclei of the embryonic notochord cells. The idea that cell death could be a fundamental inherited process was first proposed more than century later by Lockshin and Williams. They proposed that rather than a sporadic event, cell death appears in defined spatiotemporal windows during development [1]. In 1972, Kerr et al. used the term "apoptosis" meaning "to fall away from" (apo = from, ptosis = a fall), previously used to describe the falling of leaves in autumn to describe a relatively conserved set of morphological features

observed in a wide variety of cell types during physiological episodes of cell death [2]. About 12 different types of programmed cell death (PCD) have been described to date, depending on the morphological features and the molecular pathways that lead to the execution of the PCD. Apoptosis, also called programmed cell death type I, is an inherited metabolically active process during which the cell dies without induction of inflammatory response. Cells undergoing apoptosis exhibit typical morphological features. Apoptotic cells appear to be shrunken and rounded shaped, without any more pseudopodia like cytoplasmic extensions. At the cytoplasmic level, mitochondria undergo fragmentation with a concomitant loss of their transmembrane potential ($\Delta\Psi$m) [3, 4]. At the nuclear level, apoptosis is characterized by typical chromatin condensation and fragmentation giving rise to pyknotic nuclei, which can be easily observed using specific dyes such as DAPI or Hoescht. Chromatin fragmentation appears to be induced by intracellular endonucleases such as caspase-activated deoxyribonuclease (CAD) and Endonuclease G (EndoG) which preferentially cut DNA strands between nucleosomes resulting in the typical "ladder pattern" observed by electrophoresis [5].

An important feature of apoptosis is the absence of inflammatory response. Indeed, the apoptotic cell maintains its plasma membrane integrity during the whole cell death process, thus preventing the intracellular proteins to interact with surrounding cells. However, phosphatidylserine (PS), an anionic phospholipid usually found at the inner leaflet of the bilayer, is exposed outwardly in apoptotic cells [6]. This morphological feature allows macrophages to detect these cells via specific PS receptors, which is then followed by rapid internalization and phagocytosis.

In vertebrates, there are two main molecular cascades for apoptosis induction [7]. The first one, called the extrinsic pathway, activates cell death by the transduction of external death signals through plasma membrane death receptors. The second one is called the intrinsic (mitochondrial) apoptosis pathway, which essentially leads to the mitochondrial outer membrane permeabilization (MOMP) and the release of apoptotic agents. Although presented at first as individual pathways, these cascades are actually interconnected. Here, we will describe the current knowledge related to the role of the apoptosis during zebrafish development.

2. The apoptotic machinery of the zebrafish embryo

2.1. Caspases

Caspases (for Cysteine ASPartate proteASE) are intracellular cysteine proteases belonging to the family of the interleukine-1β converting enzymes (ICE) family of proteases [8]. Members of the caspase family, share similar 3D conformation and are synthesized as inactive precursors called zymogens (or pro-caspases) containing a prodomain, composed of a p20 large subunit and a p10 small subunit. Caspase activation is achieved by proteolytic cleavage between the large and small subunits and removal of the N-terminus prodomain. This post-translational modification leads to a new conformational state in which caspase homodimers are fully active. The p20 subunit contains the active site of the enzyme harboring a 'QACXG' pentapeptide motif [9]. Although caspases are primarily cytosolic, they can also be found at mitochondrial and endoplasmic reticulum (ER) membranes. Caspases have been divided into three groups: interleukin activating caspases and two additional subgroups participating in the initiation and the execution of the apoptosis, respectively.

To date, 17 caspases family members have been identified in zebrafish, including initiator and effector caspases (**Table 1**). Initiator Caspases (2, 8, 9 and 10) are characterized by the presence of a long N-terminal prodomain. Zebrafish genome contains one ortholog for each of the three Caspases 2, 9 and 10, genes with conserved synteny with the human genome [10]. It also encodes for three Caspase 8 homologous genes. Caspases 8 and 10 possess two death effector domain (DED) domains in the N-terminus whereas Caspases 2 and 9 contain one caspase recruitment domain (CARD). These domains interact with adaptor proteins and are crucial for caspase activation. Importantly, Caspase 8 was identified as an actor of the death receptor pathway (cf. Section 2.3) whereas Caspase 9 belongs to the mitochondrial apoptosis pathway. In zebrafish, Caspases 2 and 8 have been identified as important regulators of vascular development. Indeed, silencing of tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) expression using morpholinos led to the aberrant activation of Caspases 2 and 8 (but not of Caspase 3), resulting in apoptosis of endothelial cells [11]. Furthermore, Caspase 8 was found to be a downstream effector of the Yaf2 apoptosis regulator, a zinc finger-containing protein shown to interact with the DED domain of Caspase 8 and to inhibit apoptosis. Injection of zebrafish embryos with *yaf2* targeting morpholino did not affect gastrulation but compromised somitogenesis, which could be rescued by inhibiting Caspase 8 [12].

Effector caspases are characterized by a short N-terminus end devoid of a recruitment domain. They are activated *via* proteolytic cleavage by initiator caspases. Among effector caspases, Caspase 3 is critical for the execution of apoptosis, being activated by both, Caspases 8 and 9. In addition, Caspase 3 is capable of feed-back self-activation, thus accelerating apoptosis. Caspase 3, by targeting a wide range of vital cellular components, behaves actually as a genuine apoptosis executor. Activation of Caspase 3 is often considered as a no-return point of apoptosis. Among the substrates cleaved by Caspase 3, are found cytoskeleton proteins, anti-apoptotic factors, metabolic enzymes and several nucleases. For example, proteolytic cleavage of the inhibitory domain of CAD endonuclease (ICAD) leads to CAD activation and subsequent DNA fragmentation, a typical feature of the apoptotic process. The zebrafish genome encodes two Caspase 3 homologs, namely Caspases 3a and 3b [13, 14]. In fact, Caspase 3 activity has been first assessed in stressful conditions following cycloheximide or staurosporine treatments at mid-gastrula stage. In these conditions embryonic development is rapidly blocked, with Caspase 3 being activated within 8 h [15]. Furthermore, Yamashita and colleagues generated a transgenic zebrafish strain expressing full length pro-Caspase 3 to study the role of Caspase 3 in embryonic development. Indeed, these transgenics exhibited a marked increase in the number of apoptotic cells specifically in the retina, the notochord, the heart and the yolk sac, suggesting an essential role of Caspase 3 in numerous morphogenetic processes. Interestingly, silencing of *caspase 3* using specific morpholinos did not lead to any significant developmental defects, suggesting some redundancy with other effector caspases, such as Caspases 6 and 7 [16].

In addition, the zebrafish genome encodes for two caspases belonging to the interleukin activating caspases. These caspases known as Caspy (Caspy and Caspy2) contain N-terminal pyrin domains [17]. In the case of Caspy, the pyrin domain was found to be essential for its interaction with the apoptosis-associated speck-like protein containing a CARD (zAsc). In effect, zAsc binding to Caspy led to its activation and apoptosis execution *in cellulo*. In zebrafish, both genes are specifically expressed in the pharyngeal arches, *caspy* silencing resulting in developmental defects in this particular region.

Type	Mammalian protein	Zebrafish homologs	Accession number
Death receptor ligands	TNF (TNFSF2)	Tnfa (Tnf1)	NM_212859
		Tnfb (Tnf2)	NM_00102444
	CD95/FasL (TNFSF6)	Faslg (Fas ligand)	NM_001042701
	Apo2L/TRAIL (TNFSF10)	Tnfsf10 (Tnfsf10l2)	NM_001002593
		Tnfsf10l (DL1a)	NM_131843
		Tnfsf10l3 (DL1b)	NM_001042713
		Tnfsf10l4 (DL3)	NM_001013283
	APP	Appa	NM_131564
	TL1A	si:ch211-158d24.4 (tnfsf15)	NM_001123259
Death receptor	TNFR1 (TNFRSF1A)	Tnfrsf1a	NM_213190
	CD95/Fas (TNFRSF6)	Fas	XM_021467407
	TNFRSF10A and B (DR4 and DR5)	Hdr (ZH-DR)	NM_194391
	TNFRSF10A and B (DR4 and DR5)	Tnfrsfa (OTR)	NM_131840
	TNFRSF21 (DR6)	Tnfrsf21 (DR6)	NM_001042688
Adaptor protein	FADD	Fadd	XM_001923858
Initiator caspases	Caspase 2		NM_001042695
	Caspase 4	Caspy	NM_131505
		Caspy 2	NM_152884
		Caspase b, like	NM_001145592
		zgc:171731	NM_001109712
	Caspase 8	Caspase 8a	NM_131510
		Caspase 8 l1	NM_001098619
		Caspase 8 l2	XM_680338
Effector caspases	Caspase 3	Caspase 3a	NM_131877
		Caspase 3b	NM_001048066
	Caspase 6	Caspase 6a	NM_001020497
		Caspase 6b	NM_001005973
		Caspase 6c	NM_001039980
	Caspase 7	Caspase 7a	NM_001020607
Inhibitor of caspases	c-FLIP (c-FLAR)	Cflara (Cflar)	NM_001313772
	Birc 2	Birc 2, IAP 1	NM_194395
	Birc 4	Birc 4, XIAP	NM_194396

Type	Mammalian protein	Zebrafish homologs	Accession number
	Birc 5	Birc 5a, Survivin	NM_194397
		Birc 5b, Survivin 2	NM_145195
	Birc 6	Birc 6	XM_009293036.3
	Birc 7	Birc 7	NM_001098768
	Hsp 70	Hsp70	AB062116.1
BCL-2 family	Bcl-xL	zBlp1	NM_131807
(BCL-2 like)	Bcl-2	zBlp2	NM_001030253
	Mcl-1	Mcl-1a	NM_131599
		Mcl-1b	NM_194394
	Nrh, Bcl-2 l10	Nrz	NM_194398
(BAX-like)	Bax	Baxa, zBax1	NM_131562
		Baxb, Zbax2	NM_001013296
	Bok	Boka	NM_001003612
		Bokb	NM_201185
	—	Bcl-wav, Bcl-2 l16	NM_001172402
	Bcl-2 l13, Bcl-rambo	Bcl-2 l13	NM_001044891
(BH3-only)	Bad	Bada	XM_005161364
		Badb	NM_001270595
	Bbc3, Puma	zPuma, Bbc3	NM_001045472
	Noxa, Pmaip1	zNoxa, Pmaip1	NM_001045474
	Bim	zBim, Bcl2-l11	NM_001135791
	Bid	zBid	NM_001079826
	Bik	zBik	NM_001045038
	Bmf	zBmf1	NM_001045224
		zBmf2	NM_001045473
	Bnip1	Bnip1a	XM_684156
		Bnip1b	XM_001333689
	Bnip2	Bnip2	NM_201218
	Bnip3l	Bnip3la, Nix	NM_001012242
		Bnip3lb, Nip3a	NM_205571
	Bnip4l	Bnip4l, Nip3b	NM_212693
		Bnipl	NM_001128394

Type	Mammalian protein	Zebrafish homologs	Accession number
MIRAF*	Cytochrome C	Cytochorome C, Cycsb	NM_001002068
	EndoG	EndoG	NM_001024214.1
	AIF	AIF, Aifm1	NM_200102.2
	Smac/Diablo	Diabloa	NM_200346
		Diablob	NM_001243034
	HtrA2/Omi	LOC110437853	XM_021472675.1
Others	Apaf-1	Apaf-1	NM_001045243
	P53	p53, TP53	NM_131327

Accession numbers from NCBI database were presented on the left.

*MIRAF, mitochondria released apoptotic factors.

Table 1. Summary table of apoptosis-associated genes found in the zebrafish genome.

2.2. Caspase inhibitors

Since the discovery of viral caspase inhibitors, it became clear that multicellular organisms were also able to make their own caspase inhibitors. These proteins called inhibitors of apoptosis proteins (IAPs) are characterized by one or more baculoviral IAP repeats (BIR), allowing them to prevent caspase activation and apoptosis. The IAPs can also possess a RING domain with an ubiquitin-ligase activity at their C-terminus end. This feature allows the IAPs not only to block caspase activity but also to promote their degradation by the proteasome [18]. It should be noted that six IAP proteins are found in zebrafish [19]. Zebrafish IAPs including survivins (BIRC5a and BIRC5b) appear to play a role in embryonic development. Indeed, the knockdown of *birc5a* leads to multiple defects including in the nervous system, the cardiovascular system and the hematopoietic system. Interestingly, this phenotype was rescued by ectopic expression of both *birc5* paralogs, suggesting the existence of functional redundancy during embryogenesis [20, 21].

Another well characterized caspase inhibitor is the Hsp70 chaperone. Actually, under stressful conditions, the cell can protect itself from the uncontrolled activation of the apoptosis by increasing Hsp70 levels. This protein can bind to and block the recruitment of initiator Caspase 9 into the apoptosome complex [22]. In zebrafish, injection of *hsp70* targeting morpholinos resulted in "small eye" phenotype. Close analysis of this phenotype identified a significant increase in apoptotic cells specifically in the developing lens of the zebrafish embryo [23, 24].

Cellular FLICE-like inhibitory protein (c-FLIP) is another example of a cellular caspase inhibitor. This structural analog of Caspase 8 is devoided of proteolytic activity and, is able to bind to DD and prevents the activation of Caspase 8 downstream of the death receptors pathway [25]. c-FLIP is specific to the vertebrate lineage. The knockdown of *c-flip* in zebrafish results in important cardiovascular abnormalities, including cardiac edema and irregular blood flow consecutive to the formation of blood clots in the vessels [26].

2.3. Molecular actors linked to the death receptor pathway

The cell-extrinsic (or death receptor) pathway of apoptosis is activated by the binding of extracellular ligand proteins belonging to the tumor necrosis factor (TNF) superfamily to

specialized receptors, the death receptors called TNF receptors [27]. In mammals, six death receptors have been characterized together with five "death-inducing" ligands. The death receptors are characterized by at least one extracellular cystein-rich domain (CRD) allowing the recognition between the ligand and the receptor and by an intracellular conserved domain called the death domain (DD). Activation of death receptor pathway induces clustering of the receptors through their pre ligand-binding assembly domain (PLAD) [28]. This clustering triggers the recruitment of adaptor proteins such as Fas-associated protein with death domain (FADD) which interact with the DD of the receptor but also with initiator caspases (Caspases 8 and 10) thus forming the death-inducing signaling complex (DISC) [29]. DISC formation then induces activation of initiator caspases leading to the activation of effector caspases and apoptosis execution [30]. DISC-dependent caspases activation can be inhibited by c-FLIP [25].

In zebrafish, on the basis of phylogenetic analysis, orthologs of the five death receptors ligands have been identified (**Table 1**). CD95/FasL, APP and TL1A each possess one zebrafish ortholog (*faslg*, *appa* and *158d24.4*, respectively) while TNF possesses two orthologs (TNFa and TNFb) and Apo2L/TRAIL five of them (TNFSF10L, TNFSF10L2, TNFSF10L3 and TNFSF10L4) [31, 32].

The genes encoding death receptors have also been identified in zebrafish. Based on the presence and organization of their CRD and DD, clear orthologs of TNFR1 (TNFRSF1A), CD95/Fas (Fas) and DR6 (TNFRSF21) were characterized. Notably, a selective interaction between TNFa and TNFRSF1 were confirmed by immunoprecipitations [31]. Two other DD-containing receptors, HDR and TNFRSFA, have been described in zebrafish [33, 34]. Extracellular domains of these receptors are close to the one of CD95/Fas but their DD are more similar to DR4 and DR5 DD. However, the observation that HDR and TNFRSFA both bind three orthologs of Apo2L/TRAIL (TNFSF10L, TTNFSF10L2 and TTNFSF10L3) and that they are required for apoptosis induced by these ligands in zebrafish embryos strongly suggests that HDR and TNFRSFA are in fact orthologs of DR4 and DR5 [31]. Finally, so far, no zebrafish ortholog of DR3 have been characterized but the existence of an ortholog of TL1A suggests that a zebrafish DR3 will be identified shortly.

In addition to death receptors and their ligands, the components of DISC are also conserved in zebrafish. A clear ortholog of FADD, containing a DD and a death effector domain (DED), has been identified as well as an ortholog of Caspase 8, *casp8a* [31, 35]. The latter possesses an N-terminal DED which allow association with FADD and a QACQG active-site motif that is characteristic of Caspases 8 and 10 in mammals. Caspase 8a and FADD are both required for apoptosis induced by Apo2L/TRAIL orthologs in zebrafish embryos. Moreover, Caspase 8a is functionally conserved as it restores death receptor-induced apoptosis in mouse cells lacking endogenous Caspase 8 [35]. Two other genes related to *casp8a* exist (*casp8l1* and *casp8l2*) but their possible involvement in the cell-extrinsic pathway is unclear. Indeed, Caspase 8l1 has a QACQG active-site motif but no DED whereas Caspase 8l2 possesses an N-terminal DED but its active site is similar to the one of Caspase 2. Finally, a zebrafish ortholog of c-FLIP, referred to as Cflar, able to inhibit apoptosis induced by Apo2L/TRAIL orthologs have also been described [31].

In mammals, the cell-extrinsic apoptosis pathway is essential for the functioning of the immune system. However, this has not been clearly established in zebrafish as yet. One study revealed that apoptosis is important for T and B cells homeostasis as overexpression of Bcl-2 in these cells increased their number, but this work did not show a role for the extrinsic pathway

in lymphocyte homeostasis [36]. In contrast, the death receptor HDR appears to be involved in red blood cells homeostasis. Indeed, HDR is specifically expressed in hematopoietic lineage and its inhibition, using either a dominant negative mutant or antisense morpholinos, leads to abnormal accumulation of erythroid cells [34, 37].

During early development, *hdr*, *tnfrsfa* and *fadd* are expressed in the notochord suggesting that they might play a role in this structure [31]. Fas and FasLg are also present in developing notochord and their knockdown *via* morpholinos leads to prolonged expression of notochord specific genes and to an abnormally enlarged notochord at 4 days post-fertilization (dpf). As apoptosis plays a role in notochord regression, this suggests that extrinsic pathway may be involved in this process [38].

During development, TNFRSFA and its ligands TNFSF10L2 and TNFSF10L3 are expressed in particular in neuromasts that contain hair cells which turnover is regulated by apoptosis [31, 39]. Finally, the death receptor pathway seems to be involved in zebrafish eye development as FADD is required for slowing cell growth during this process [40].

2.4. Molecular actors linked to the intrinsic pathway

The intrinsic pathway of apoptosis also called the mitochondrial pathway can be induced by various signals including DNA damage, chemotherapy, viral infection or growth factors deprivation. The mitochondrial pathway of apoptosis is mainly controlled by the Bcl-2 family of proteins, which is described in more detail in the next chapter. The mitochondrion is a membrane-enclosed organelle found in most eukaryotic cells [41]. The diameter of these organelles falls under the 0.5–10 μm range. Mitochondria are often referred as the "power-house" of the cell because they generate most of the cell's supply of adenosine triphosphate (ATP). In addition to their bioenergetic function, mitochondria are involved in a number of other processes, such as signal transduction, cell differentiation, cell cycle and cell growth [42]. Mitochondria are also major "decision centers" for the execution or prevention of apoptosis. Indeed, a number of pro-apoptotic molecules appear to be stored in the existing space between the inner mitochondrial membrane (IMM) and the OMM. At the mitochondrial level, the induction of apoptosis leads to the OMM permeabilization, which leads to the irreversible release into the cytosol of pro-apoptotic factors that promote caspase activation, DNA fragmentation and ultimately the death of the cell. Belong to this toxic molecular "cocktail" among others: Cytochrome C, AIF, EndoG, Smac/Diablo and HtrA2/Omi.

2.4.1. Mitochondria released apoptotic factors

Cytochrome C is a small heme protein (approximately 12 kDa), exhibiting a positive net charge, located in the intermembrane space (IMS) where it can be loosely attached to the IMM. Cytochrome C is synthesized inside the cytosol, and subsequently transported into the IMS. Bioenergetically, Cytochrome C is a component of the mitochondrial electron transport chain. The heme molecule of cytochrome C accepts electrons originating from complex III and transfers them to the cytochrome oxydase complex, thus cytochrome C is indispensable for the oxidative phosphorylation and the maintenance of cellular energy fluxes. Cytochrome C plays an additional role in the context of apoptosis, as it is now well established by a large

number of *in vitro* and *in vivo* studies [43–46]. The release of Cytochrome C is a rapid and complete process, irrespective of the intensity of the death-inducing signal or the temperature, indicating that this is a non-enzymatic phenomenon obeying an "all or nothing" law. When released in the cytosol, Cytochrome C interacts with the adaptor protein apoptosis protease activating factor (Apaf-1) in presence of ATP. Apaf-1 was first characterized by Wang and collaborators [47]. It is a 130 kDa multidomain protein, containing a CARD domain at its N-terminus end, sharing homology with the CARD domain of Caspase 9, an ATPase domain as well as two WD-40 repeats at the C-terminus end. The Wang laboratory discovered the existence of a multiprotein complex called the apoptosome, comprising Cytochrome C, Apaf-1 and Caspase 9, which was found to be able to activate Caspase 3 [48]. The apoptosome was crystallized by Acehan and collaborators in 2002 [49]. Although the existence of the zebrafish apoptosome has not been directly demonstrated, its genome encodes for all functional components of this complex. Furthermore, treatment with drugs converging toward the intrinsic pathway was found to lead to Caspase 3 activation [50, 51].

Apoptosis-inducing factor (AIF) is a 57 kDa flavoprotein with NADH oxidase activity which is located in the mitochondrial IMS [52, 53]. As Cytochrome C, AIF is encoded by a nuclear gene, which is imported into the mitochondria after being synthesized in the cytosol. In response to diverse death stimuli, AIF is released into the cytosol and transferred to the nucleus where it binds to chromatin [54]. The binding of AIF to DNA induces chromatin peripheral condensation and subsequent fragmentation. Wang and colleagues showed that this process is due to AIF-dependent activation of endogenous nucleases such as endonuclease G and CAD. AIF is unable to induce the fragmentation of the DNA on its own. To this end, AIF needs the help of endonuclease G. EndoG is a mitochondrial nuclease of 30 kDa which is required for the replication of the mitochondrial chromosome. During apoptosis, EndoG is released from the mitochondria into the cytosol and subsequently enters into the nucleus. In this compartment, EndoG cleaves the DNA into nucleosomal fragments [55]. AIF and EndoG orthologs are both expressed during zebrafish embryonic development but their functional implications remain to be analyzed. Second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI (Smac/Diablo) is a mitochondrial protein, which resides in the intermembrane space [56, 57]. The human gene is called *smac* whereas the mouse ortholog is called *diablo*. Smac/Diablo is synthesized as a precursor protein containing a 55 residue N-terminal mitochondrial targeting sequence. During the translocation of Smac/Diablo to the mitochondria, this sequence is cleaved which uncovers an IAP-binding motif (IBM) required for apoptotic activity. Indeed, in the presence of an apoptotic stress, the mitochondria release Smac/Diablo in the cytosol where it participates indirectly in the activation of caspases by binding IAP proteins. The binding of Smac/Diablo to IAP disrupts IAP-Caspase interactions. Thus, released caspases can then be activated and execute the cell death program. The Smac/Diablo-IAP complex formation appears to be a regulated process since IAP can ubiquitinate Smac/Diablo and drive it for proteasomal degradation [18]. In zebrafish, Smac/Diablo gene is mainly expressed in the late developmental stages with the most prominent expression in the heart, the lens and the liver. However, the possible implication of this IMS factor in the morphogenesis of these organs remains unknown.

High temperature requirement A2 (HtrA2/Omi) is a heat shock protein first identified in *Escherichia coli* [58]. Its ortholog in mammals, called Omi was initially described as an ER protein

[59, 60]. However, it seems that this protein is mainly mitochondrial in non-apoptotic cells [61]. When the OMM is permeabilized, Omi is released into the cytosol where it binds to IAP and irreversibly inhibits its activity by proteolytic cleavage [57]. Thus, it seems that Omi is a more potent inhibitor of IAP than Smac/Diablo which blocks IAP reversibly. Currently, there is no data about the possible implication of HtrA2/Omi functional implication in zebrafish have not been assessed.

2.4.2. Bcl-2 family of proteins

The Bcl-2 family of proteins is a group of intracellular eukaryotic proteins best known for their implication in MOMP. The founding member of this family, the *bcl2* (B-cell lymphoma/leukemia 2) gene was discovered in a study on a chromosome translocation frequently observed in human B-cell follicular lymphomas. Tsujimoto and colleagues showed that the translocation between chromosome 18 (q21) chromosome 14 (q32), t (14;18), results in the relocation of the *bcl2* ORF downstream of the enhancer promoter region of the *igh* heavy chain immunoglobulin gene [62–64]. This translocation results in transcriptional upregulation of *bcl2* gene expression [65]. The Bcl-2 family comprises proteins with antagonistic functions with respect to apoptosis regulation. Structurally, all Bcl-2 proteins contain in their primary structure one or more conserved Bcl-2 homology (BH) domains. Based on this criterion, three subgroups have been identified: (1) the anti-apoptotic multidomain members containing all four BH domains (BH1–4); (2) the pro-apoptotic members containing three BH domain (lacking BH4) and (3) the pro-apoptotic BH3-only members containing only the sole BH3 domain. In addition, Bcl-2 proteins may contain a transmembrane anchoring domain (TM domain) at the C-terminus end. Bcl-2 multidomain pro-apoptotic members (Bax and Bak) are the effectors for MOMP. Through their oligomerization, they form pores at the MOM and promote the release of apoptotic factors including Cytochome C, AIF and EndoG [66]. Anti-apoptotic Bcl-2 members block their activity and promote cell survival, whereas BH3-only proteins play the role as intracellular judges as they can inhibit anti-apoptotic members and/or promote Bax/Bak oligomerization.

Bcl-2 homologs of all three subgroup of Bcl-2 family have been identified and molecularly characterized in zebrafish [67–69]. Due to genomic duplication, the zebrafish genome possesses several Bcl-2-related paralogs including Mcl-1a, Mcl-1b, Bax1, Bax2, Boka, Bokb and Bmf1 and Bmf2. Interestingly, the ortholog of the *bak* gene has been lost in the teleost lineage. Instead the zebrafish genome harbors *bcl-wav* (an acronym for Bcl-2 homolog found in water living anamniotes), a *bcl-2* homolog only found in fishes and anurans [70]. With the exception of the BH3-only protein Bik, all Bcl-2 related genes in zebrafish are maternally inherited since their corresponding mRNAs were detected before the mid-blastula transition [67].

Of note during zebrafish early development physiological apoptosis is not observed suggesting that at least some of the Bcl-2 family members may have additional non-apoptotic roles during early embryogenesis. In this respect, Zhong and colleagues recently demonstrated that the zebrafish ortholog of the BH3-only protein Noxa (zNoxa) not only controls apoptosis during late stages of gastrulation but also plays role in cell cycle in the developing blastula [71]. Indeed *znoxa* knockdown led to a significant decrease in the number of mitotic cells. This phenotype seems to be dependent on the Wnt signaling pathway since *znoxa* knockdown led to increase of *zwnt4b* expression. In addition silencing of *zwnt4b* rescued zNoxa phenotype and restored a WT count of cells in G2/M phase.

Using the zebrafish model, we demonstrated that a Bcl-2 homolog, referred to as Nrz (Nr-13 ortholog in zebrafish), is critical during the early stages of zebrafish development [72]. In zebrafish, Nrz protein possesses a dual subcellular localization at the ER and the mitochondria [73]. Its functional invalidation causes embryo development arrest followed by detachment of the entire blastomeres from the yolk sac. By performing a series of time-lapse and confocal microscopy experiments, we demonstrated that this phenotype is due to the premature formation of the actin-myosin contractile ring, a supramolecular structure, which squeezes and halves the embryo at the level of the margin. Furthermore, by using single subcellular localization (SSL) Nrz mutants, we showed that the ER-resident Nrz but not mitochondrial-resident Nrz was critical for its physiological function. Indeed, at the ER membrane, Nrz interacts with the Ca^{2+} channel inositol trisphosphate receptor type 1 (IP_3R1) *via* its BH4 domain [73, 74]. In this way, Nrz slows down the release of Ca^{2+} into the yolk sac, which consecutively allows controlling the formation of the contractile actin-myosin ring *via* the Calmodulin-MLCK pathway. Overall, our results highlighted for the first time that a Bcl-2 family member is able to orchestrate cellular migration events by controlling intracellular Ca^{2+} fluxes.

In addition, we identified the new Bcl-2 family member Bcl-wav [75]. The *bclwav* gene is expressed throughout zebrafish early development; it encodes a pro-apoptotic Bcl-2 family member with strict mitochondrial localization. However, *bclwav* silencing in zebrafish causes a specific apoptosis independent phenotype at 24 hpf. Macroscopically, this phenotype is characterized by an embryo anterioposterior axis reduction as well as notochord deviation. Using time-lapse microscopy, we demonstrated that this phenotype affects the convergence and extension movements which underlie the establishment of the embryonic axes. Indeed, in *bclwav*-silenced embryos, mesodermal cells migrated erratically compared to cells from control embryos, which moved in a coordinated fashion. *In vivo* analysis of the actin cytoskeleton revealed that these migration defaults correlated with randomization of F-actin protrusion dynamics. Interestingly, *bclwav* silencing was correlated with a decrease of mitochondrial Ca^{2+} levels and concomitant increase of cytosolic Ca^{2+}. Together these results indicated that Bcl-wav controls the dynamics of the actin cytoskeleton by regulating intracellular Ca^{2+} homeostasis at the mitochondrial level. Indeed, at the mitochondria, Bcl-wav interacts with the voltage-dependent anion channel (VDAC) channel and enhances mitochondrial Ca^{2+} uptake, which in turn controls actin polymerization and cell migration. It is important to note that, *mitochondrial calcium uniporter* (*mcu*) knockdown phenocopies *bclwav*-silenced embryos. Indeed, MCU downregulation leads to decreased mitochondrial calcium uptake and impaired actin dynamics giving the first insights into the critical role of the mitochondrial Ca^{2+} oscillations in the vertebrate development [70].

Put into a broader context, the results demonstrate that members of the Bcl-2 family are able to control cell migration in a calcium-dependent manner *via* their direct interaction with intracellular Ca^{2+} channels independently of their involvement in the regulation of cell death [76].

2.4.3. P53

P53 is a transcription factor considered as the main tumor suppressor regulating cell fate decisions. Indeed, p53 is the most frequently mutated and/or inactivated gene in human cancer modulating cell responses to DNA damage, oncogenic signaling and hypoxia in order to preserve genome integrity. The zebrafish p53 ortholog is highly conserved with 48% of sequence

identity compared to the human sequence [77]. P53 is highly and ubiquitously expressed during early zebrafish embryo development, then predominantly expressed in the brain during the pharyngula stage before to decrease in expression after 48 hpf [77–79]. P53 tumor suppressor activity has been confirmed in the zebrafish model by the characterization of a p53 mutant harboring a missense mutation in the DNA-binding domain, M214K, leading to the development of multiple organ tumors at around 8.5 months [80]. The mechanism of action of p53 has been widely studied in mammals and similar results have been obtained in the zebrafish model [81]. During different cell stresses, p53 expression is stabilized leading to its activation and accumulation in the nucleus, and subsequently to cell cycle arrest and the intrinsic pathway of apoptosis [82].

Its role in DNA damage and apoptosis has been well studied in the zebrafish model [82]. It has been shown that increased DNA damage leads to the stimulation of p53 transcription and an increase of p53 protein level [79]. In addition, knockdown of *p53* decreases apoptosis induced by different stimuli including gamma and UV irradiation, camptothecin treatment or altered DNA replication [83–85].

Finally, an undesirable effect of p53 activation-induced cell death has been characterized and associated with off-target effects triggered by morpholino antisense oligonucleotides technologies. These off-target effects have been assimilated to p53 signaling pathway-dependent apoptosis [86, 87]. Indeed, the most commonly off-target reported phenotype is characterized by an increase of neural cell death at 22 hpf [86]. Even if the precise molecular mechanism is still unclear, it has been attributed to the activation of p53 leading to the apoptotic cell death. Indeed, the role of p53 in this phenotype has been shown by the characterization of *smo* and *wnt5* morphant embryos, where simultaneous knockdown of p53 in these embryos rescued neuronal apoptosis [87]. These results highlight the extremely cautious, which has to be employed when potential actors of the apoptotic program are studied by the morpholino strategy. In addition to the use of different morpholino sequences targeted, the same gene and crucial rescue experiments, co-expression of p53 morpholino is now commonly used to discern between gene-specific and off-target effects [19]. Indeed, p53 is not required for proper early embryos development and p53 morpholino does not interfere with other gene-specific phenotypes [87, 88].

3. Developmental control of apoptosis in the early zebrafish embryo

Following fertilization and during early stages of embryonic development, embryo relies entirely on the maternal inherited mRNAs and proteins which were accumulated during oogenesis. After several synchronous divisions, which lack G1 and G2 phases, the cell cycle slows down and divisions become asynchronous. This step, referred to as the mid-blastula transition (MBT), corresponds to the beginning of the expression of zygotic genes. Ikegami and colleagues first noticed that zebrafish embryos treated before the MBT with microtubule destabilizing agent nocodazole or DNA-damaging molecules such as camptothecine, hydroxyurea or aphidicoline did not result in direct apoptosis activation. Instead, the cell cycle was arrested with the apoptotic program being executed several hours later, during the mid-gastrula stage [50, 51]. This phenomenon is not restricted to zebrafish as a similar

apoptotic control also operates in the Xenopus embryo [89]. These observations suggested that key molecular components of the apoptotic program were either missing or inactivated during early development. In this respect, one report using a proteomics approach identified that Apaf-1 was missing before the MBT suggesting that a functional apoptosome may be set up after the MBT transition [90]. However, these later data do not explain why inhibition of protein synthesis using cycloheximide is not able to slow the apoptotic program in the zebrafish gastrula [15]. Furthermore, our laboratory demonstrated that ectopic expression of zBax through recombinant mRNA injection in one cell stage embryos actually led to $\Delta\Psi$m loss and Caspase 3 activation as early as the blastula stage [91]. Importantly, this apoptotic phenomenon specifically occurs in an extraembryonic structure referred to as the yolk syncytial layer (YSL). The YSL results from the fusion of blastomeres physically connected with the yolk cell by cytoplasmic bridges [92]. Fusion of margin blastomeres with the yolk leads to the release of cell nuclei and other cellular components including a dense network of active mitochondria interconnected with ER membranes. Purified YSL mitochondria can undergo MOMP and Cytochrome C release. This was demonstrated by performing *in vitro* Cytochrome C release assay using recombinant human truncated Bid protein. Indeed, Bid is a BH3-only protein which once cleaved by Caspase 8 translocates to the MOM and activates Bax oligomerization. Thus it is tempting to speculate that at least at the level of the YSL mitochondria harbor sufficient amount of Bax in order to initiate MOMP following BH3-only stimulus.

Altogether these results showed that zebrafish early embryo possesses a functional apoptotic machinery. Thus the tight apoptotic control observed by Ikegami et al. may be exerted at the post-translational level through protein-protein interactions. In this respect, Kratz and colleagues demonstrated that manipulation of the ratio between pro- or anti-apoptotic Bcl-2 proteins determines the capacity of early zebrafish embryo to undergo apoptosis. Notably, overexpression of BH3-only or zBax paralogs induced rapid Caspase 3-dependent cell death whereas co-expression of Bcl-2-related anti-apoptotic members effectively counteracted early embryo mortality [67].

4. Conclusion

Apoptosis represents a key cellular process that maintains tissue homeostasis and shapes the embryo. Impairment or *a contrario* overactivation of cell death often leads to severe developmental abnormalities and lethal phenotypes. Thus, the tight spatiotemporal control over apoptosis induction is critical for orchestrating embryonic development. The fact that zebrafish genome encodes for the majority of apoptosis actors found in the human genome makes zebrafish a valuable model for understanding the contribution of apoptosis regulators during embryonic development in vertebrates. The use of antisense chemically modified nucleotides, most notably morpholinos, allowed to assess the implication of many apoptosis regulators in the developmental process. However, the possible off targeting and unspecific activation of the p53 pathway can be a drawback in some instances. In this respect, the development of new genome editing approaches such as CRISPR/Cas9 will allow in the near future to assess the precise role of each and every member of the cell death machinery during embryogenesis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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