Communicative & Integrative Biology 7:1, e28008; February; © 2014 Landes Bioscience

Nrz but not zBcl-xL antagonizes Bcl-wav pro-apoptotic activity in zebrafish

Julien Prudent[1], Germain Gillet[1], and Nikolay Popgeorgiev[1*]

[1]Université de Lyon; Centre de Recherche en Cancérologie de Lyon; U1052 INSERM; UMS 3453 CNRS; Université Lyon I; Centre Léon Bérard, Lyon, France

Keywords: Apoptosis, Bcl-2 proteins, zebrafish, Bcl-wav, Nrz, zBcl-xL

Abbreviations: OMM: outer mitochondrial membrane; VDAC: voltage-dependent anion channel; MCU: mitochondrial calcium uniporter

We recently identified a new highly divergent Bcl-2 related protein, named Bcl-wav, with phylogenetic pattern restricted to aquatic anamniotes. In zebrafish gastrula, *bclwav* gene silencing resulted in calcium-dependent cytoskeleton remodeling leading to convergence and extension movements defaults and abnormal orientation of the larva notochord. Beyond its function in cell migration, Bcl-wav was found to act as a pro-apoptotic Bcl-2 member inducing Bax/Bak dependent cell death. Here we show that, in zebrafish, pro-apoptotic Bcl-wav activity is selectively counteracted by the anti-apoptotic Nrz protein but not by zBcl-xL. Indeed Nrz but not zBcl-xL was able to decrease Bcl-wav dependent embryo mortality. Furthermore Nrz was able to prevent apoptosis induced by Bcl-wav ectopic expression in the embryo's head and tail. Finally co-immunoprecipitation experiments in HeLa cells showed that Bcl-wav directly interacts with Nrz. Overall these results expand our current knowledge about Bcl-2 family proteins interactome during early zebrafish development.

Bcl-2 family proteins are major regulators of the programmed cell death type I known as apoptosis. They control a critical point of no-return: the outer mitochondrial membrane (OMM) permeabilization which leads to the release of cytotoxic molecules, activation of intracellular proteases (caspases) and the death of the cell.[1] In fact Bcl-2 proteins are organized in two groups of apoptosis regulators; the pro-apoptotic Bcl-2 members, which lead to OMM permeabilization, and the anti-apoptotic ones which interact with the pro-apoptotic proteins and antagonize their activity.[2] In addition to their role in apoptosis, several members of the Bcl-2 family are involved in the control of intracellular calcium exchanges between the endoplasmic reticulum and the mitochondria.[3,4] Recently we characterized a new Bcl-2 homolog, named Bcl-wav, during early zebrafish development.[5] Knockdown experiments in zebrafish embryos demonstrated the capacity of Bcl-wav to control actin polymerization events during convergence and extension movements of the mesodermal progenitors cells which are critical for the anteroposterior axis positioning. We showed that Bcl-wav is able to control cell migration during zebrafish gastrulation by regulating mitochondrial calcium uptake via its interaction with two critical components of the mitochondrial calcium turnover: the voltage-dependent anion channel 1 (VDAC1)[6,7] and, possibly, the mitochondrial calcium uniporter (MCU).[8,9] In addition to its effect on mitochondrial calcium homeostasis Bcl-wav also acts as a bona fide pro-apoptotic Bcl-2 member as shown by its capacity to induce Caspase 3 activation both in cultured cells and in zebrafish embryos.[5] Here we compared the effects of two Bcl-2 apoptosis inhibitors: Nrz (the zebrafish ortholog of human Bcl-2l10) and zBcl-xL (the zebrafish ortholog of human Bcl-xL) on Bcl-wav-induced apoptosis. We coinjected *bclwav* mRNA in combination with *egfp, nrz* or *zbclxl* mRNA in zebrafish embryos at 1 cell stage. As shown in **Figure 1A**, ectopic expression of Bcl-wav together with EGFP, induced significant embryo mortality at 9 hours post fertilization (hpf) (percentage of embryos mortality at 9 hpf: 44%, ± 3%) compared with EGFP alone (10%, ± 4%). Interestingly Nrz, but not zBcl-xL, rescued the lethal phenotype of *bclwav* mRNA injected embryos. Indeed, Nrz was able to restore embryo viability and epiboly progression (19%, ± 3%) whereas zBcl-xL had no effect (41%, ± 10%) (**Fig. 1A**). We next used the fluorescent dye, acridine orange which allows in vivo detection of dying cells in the zebrafish larva. Acridine orange staining was performed on 24 hpf embryos expressing *bclwav* either alone or in combination with *nrz* or *zbclxl*. As expected, Bcl-wav ectopic expression induced a marked increase of the number of dying cells in the tail and in the head, compared with controls (**Fig. 1B**). Moreover, Nrz expression was able to counteract this effect, significantly reducing the number of dying cells. In contrast zBcl-xL was less efficient in this respect (**Fig. 1B**). These results suggested that Nrz may directly interact with Bcl-wav and block its pro-apoptotic activity. To confirm this view, we performed co-immunoprecipitation experiments in HeLa cells expressing Nrz and Flag-tagged Bcl-wav (Flag-Bcl-wav) proteins. As shown in **Figure 1C**, immunoprecipation

*Correspondence to: Nikolay Popgeorgiev: npopgeorgiev@gmail.com
Submitted: 11/19/2013; Revised: 01/25/2014; Accepted: 01/27/2014; Published Online: 02/06/2014
Citation: Prudent J, Gillet G, Popgeorgiev N. Nrz but not zBcl-xL antagonizes Bcl-wav pro-apoptotic activity in zebrafish. Communicative & Integrative Biology 2014; 7:e28008; http://dx.doi.org/10.4161/cib.28008

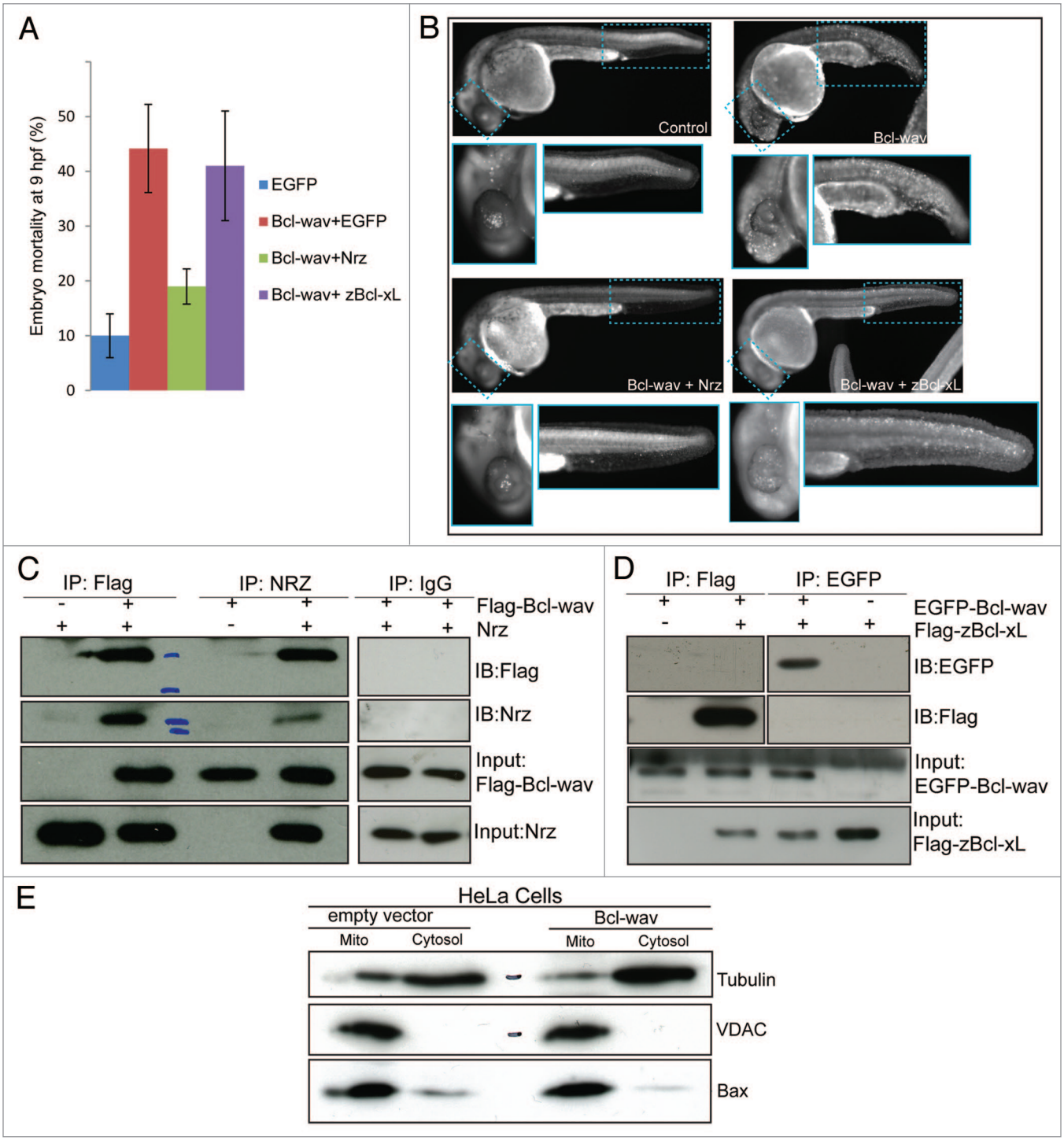

Figure 1. Nrz interacts with Bcl-wav and regulates its pro-apoptotic activity in zebrafish. (**A**) Histogram showing the percentage of embryo mortality at 9 hours post fertilization. Embryos at one cell stage were injected with *egfp* mRNA alone, *egfp* plus *bclwav* mRNAs or *bclwav* mRNA in combination with *nrz* or *zbclxl* mRNAs. Nrz overexpression rescues the Bcl-wav lethal phenotype in contrast to zBcl-xL (mean ± SD; three independent experiments). (**B**) Acridine orange cell death staining of zebrafish embryos expressing *bclwav* alone or in combination with *nrz* or *zbclxl*. *Bclwav* expression (top right panels) leads to marked increase of the number of dying cells in the tail and head regions correlated with malformation observed of these regions compared with control embryos (top left panels). *Nrz* (bottom left panels) overexpression is able to rescue this phenotype in contrast to *zbclxl* overexpression (bottom right panels). (**C**) Bcl-wav interacts with Nrz. Co-immunoprecipitation was performed with protein extracts from transfected HeLa cells with pCS2+Flag-Bcl-wav and pCS2+Nrz using anti-FLAG and anti-Nrz antibodies, respectively. Irrelevant IgG were used to verify the specificity of this interaction. (**D**) Bcl-wav does not interact with zBcl-xL. Co-immunoprecipitation was performed with protein extracts from transfected HeLa cells with pEGFP-C1-Bcl-wav and pCS2+Flag-zBcl-xL using anti-GFP and anti-FLAG antibodies, respectively. (**E**) Analysis of mitochondrial and cytosolic Bax levels in purified mitochondria from Flag-Bcl-wav expressing HeLa cells. Bcl-wav increases the mitochondrial to cytosolic Bax ratio, compared with control cells. Anti-Tubulin and anti-VDAC antibodies were used as cytosolic and mitochondrial markers, respectively.

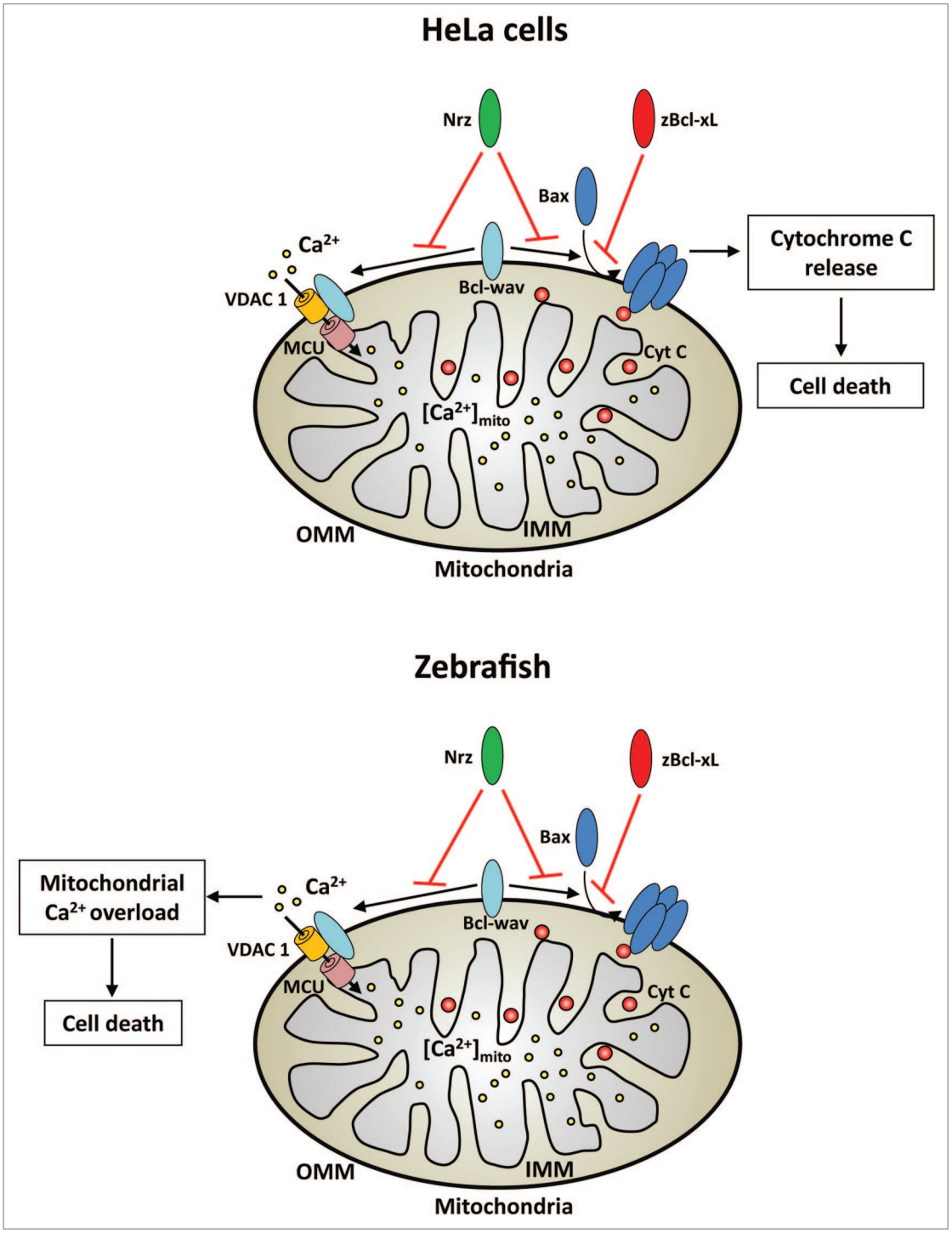

Figure 2. Schematic representation of Bcl-wav induced cell death *in cellulo* and in zebrafish embryos. In HeLa cells Bcl-wav overexpression leads to mitochondrial translocation and accumulation of Bax and subsequent cell death. This pro-apoptotic activity is inhibited by the overexpression of both anti-apoptotic proteins: Nrz and zBcl-xL. zBcl-xL failed to interact with Bcl-wav and seems to prevent apoptosis induction by inhibiting Bax oligomerization. In comparison, Nrz seems have a dual role by inhibiting zBax dependent-cell death and directly interacting with Bcl-wav. In zebrafish embryos, Nrz but not zBcl-xL is able to prevent Caspase 3 activation and cell death events at later stages suggesting that in this case apoptosis is induced in a Bax-independent manner. Nrz may possibly act via the disruption of Bcl-wav/VDAC1 complex and preventing a lethal mitochondrial calcium overload. OMM: outer mitochondrial membrane, IMM: inner mitochondrial membrane, Cyt C: Cytochrome C.

of Nrz with polyclonal anti-Nrz antibody resulted in the pull-down of Flag-Bcl-wav. Conversely Nrz was pulled-down when immunoprecipitating Flag-Bcl-wav showing that these two proteins were able to form a heterocomplex (**Fig. 1C**). However no interaction could be detected between Bcl-wav and zBcl-xL (**Fig. 1D**).

Overall the above results demonstrate that Nrz but not zBcl-xL specifically interacts with Bcl-wav and blocks its pro-apoptotic activity in zebrafish. We previously reported that Bcl-wav-induced apoptosis is Bax-dependent. Indeed Bcl-wav expression in *bax/bak* deficient mouse embryonic fibroblast cells is unable to activate Caspase 3.[5] Moreover, in HeLa cells, Bcl-wav was found to interact with Bax[5] which resulted in its mitochondrial translocation and accumulation, leading to Caspase 3 activation and subsequent cell death (**Fig. 1E**). Actually, in this particular case, Nrz as well as zBcl-xL possibly inhibited Bcl-wav pro-apoptotic activity by preventing Bax oligomerization and OMM permeabilization (**Fig. 2**). However during early zebrafish development, zBcl-xL was found unable to inhibit Bcl-wav pro-apoptotic activity in contrast to Nrz. It seems that a second process could lead to a zBax-independent cell death following Bcl-wav overexpression in developing embryos. Remarkably zebrafish gastrula is resistant to a large number of apoptotic stimuli although it harbors functional apoptosis machinery, suggesting that Bax expression and/or activity is tightly regulated at this stage.[10]

We previously reported that Bcl-wav physiological function was to regulate mitochondrial calcium pool by directly binding to VDAC1. Indeed, we showed that Bcl-wav overexpression leads to an increase of the mitochondrial calcium uptake.[5] Calcium overload is known to induce mitochondrial swelling, permeability transition and cell death independently of Bax activation.[11] Thus it is tempting to speculate that overexpression of Bcl-wav could lead to massive mitochondrial calcium entry and subsequent cell death which is inhibited by Nrz in zebrafish embryos. In this line, it can be suggested that Nrz could compete with Bcl-wav for VDAC1 binding at the mitochondria and in this way control mitochondrial calcium pool.

Interestingly, Bcl-wav still exhibits pro-apoptotic activity in *mcu* silenced zebrafish embryos or human HeLa cells suggesting that OMM permeabilization is *mcu* independent and corroborating the notion of a zBax-dependent apoptotic pathway.[5] However Bcl-wav overexpression may still induce cell death by mitochondrial calcium deregulation when zBax oligomerization is inhibited. Thus additional experiments on *zbax1* knockdown embryos should be performed to confirm the capacity of Bcl-wav to activate a zBax-independent death pathway. Additionally, experiments on *mcu/zbax1* double knockdown embryos overexpressing Bcl-wav have to be performed to confirm the capacity of Bcl-wav to induce apoptosis via these two pathways. Finally, it should be worth to shed light on the precise mechanism of Bcl-wav/Nrz interaction as well as on its significance during early zebrafish development. Together our results also suggest a new mitochondrial role of Nrz and in particular in the control of mitochondrial calcium fluxes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Région Rhône Alpes and the Fondation pour la Recherche Médicale.

References

1. Bender T, Martinou J-C. Where killers meet--permeabilization of the outer mitochondrial membrane during apoptosis. Cold Spring Harb Perspect Biol 2013; 5:a011106; PMID:23284044; http://dx.doi.org/10.1101/cshperspect.a011106
2. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 2008; 9:47-59; PMID:18097445; http://dx.doi.org/10.1038/nrm2308
3. Bonneau B, Prudent J, Popgeorgiev N, Gillet G. Non-apoptotic roles of Bcl-2 family: the calcium connection. Biochim Biophys Acta 2013; 1833:1755-65
4. Popgeorgiev N, Bonneau B, Ferri KF, Prudent J, Thibaut J, Gillet G. The apoptotic regulator Nrz controls cytoskeletal dynamics via the regulation of Ca2+ trafficking in the zebrafish blastula. Dev Cell 2011; 20:663-76; PMID:21571223; http://dx.doi.org/10.1016/j.devcel.2011.03.016
5. Prudent J, Popgeorgiev N, Bonneau B, Thibaut J, Gadet R, Lopez J, Gonzalo P, Rimokh R, Manon S, Houart C, et al. Bcl-wav and the mitochondrial calcium uniporter drive gastrula morphogenesis in zebrafish. Nat Commun 2013; 4:2330; PMID:23942336; http://dx.doi.org/10.1038/ncomms3330
6. Rapizzi E, Pinton P, Szabadkai G, Wieckowski MR, Vandecasteele G, Baird G, Tuft RA, Fogarty KE, Rizzuto R. Recombinant expression of the voltage-dependent anion channel enhances the transfer of Ca2+ microdomains to mitochondria. J Cell Biol 2002; 159:613-24; PMID:12438411; http://dx.doi.org/10.1083/jcb.200205091
7. De Stefani D, Bononi A, Romagnoli A, Messina A, De Pinto V, Pinton P, Rizzuto R. VDAC1 selectively transfers apoptotic Ca2+ signals to mitochondria. Cell Death Differ 2012; 19:267-73; PMID:21720385; http://dx.doi.org/10.1038/cdd.2011.92
8. Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, et al. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 2011; 476:341-5; PMID:21685886; http://dx.doi.org/10.1038/nature10234
9. De Stefani D, Raffaello A, Teardo E, Szabò I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 2011; 476:336-40; PMID:21685888; http://dx.doi.org/10.1038/nature10230
10. Popgeorgiev N, Prudent J, Bonneau B, Gillet G. The yolk cell of the zebrafish blasula harbors functional apoptotis machinery. Commun Integr biology 2011; 4-5:549-51
11. Tan W, Colombini M. VDAC closure increases calcium ion flux. Biochim Biophys Acta 2007; 1768:2510-5