

Molecular basis of BCL2L10/Nrh oncogenic activity in breast cancer

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Molecular basis of BCL2L10/Nrh oncogenic activity in breast cancer

Bases moléculaires de l'activité oncogénique de BCL2L10/Nrh dans le contexte du cancer du sein

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MANDATORY FEATURES

Titre Français

Bases moléculaires de l'activité oncogénique de BCL2L10/Nrh dans le contexte du cancer du sein

Mots-clés en Français

Protéines Bcl-2 - Apoptose - BCL2L10/Nrh - Récepteurs à l'IP3 (IP3R) - Calcium - Peptides

Résumé Français (1700 caractères)

L'apoptose, ou « mort cellulaire programmée », joue un rôle clé dans de nombreux processus biologiques. Les protéines de la famille Bcl-2, dont l'expression est souvent altérée dans les cellules tumorales, sont les principaux régulateurs de l'apoptose. Parmi cette famille, la fonction exacte du répresseur apoptotique Nrh, aussi appelé BCL2L10 ou Bcl-B, reste à ce jour mal comprise. Bien que son expression ne soit pas détectable dans la plupart des tissus sains, on retrouve des niveaux élevés de Nrh corrélés à un mauvais pronostique dans les cancers du sein et de la prostate.

Nous avons mis au jour un nouveau mécanisme selon lequel Nrz, l'orthologue de Nrh chez le poisson zèbre, interagit avec le domaine de liaison du ligand IP3 du canal calcique IP3R1. Il s'est avéré que la régulation négative des flux calciques par Nrz est critique lors du développement embryonnaire du poisson zèbre. Grâce à ces nouvelles données, nous avons cherché à comprendre la fonction de Nrh chez l'Homme, dans un contexte pathologique. Nous avons montré que Nrh interagit via son domaine BH4 avec le domaine de liaison du ligand du récepteur IP3R1 humain pour réguler l'homéostasie calcique et la mort cellulaire. Cette interaction définit Nrh comme la seule protéine de la famille Bcl-2 à réguler négativement la mort cellulaire exclusivement au niveau du réticulum endoplasmique. Pour aller plus loin, nous avons montré que la dissociation du complexe Nrh/IP3Rs sensibilise des cellules tumorales mammaires à l'action d'agents chimiothérapeutiques. Pour finir, nos résultats apportent une explication moléculaire sur la contribution de Nrh dans la résistance aux thérapies anti-tumorales.

Titre Anglais

Molecular basis of BCL2L10/Nrh oncogenic activity in breast cancer

Mots-clés en Anglais

Bcl-2 proteins - Apoptosis - BCL2L10/Nrh - IP3 Receptors (IP3Rs) - Calcium - Peptides

Résumé Anglais (1700 caractères)

Apoptosis, also called "Programmed Cell Death", plays a key role in many biological processes and pathologies. The B-cell lymphoma 2 (Bcl-2) proteins, whose expression is often altered in tumor cells, are the main regulators of apoptosis.

Among this family, the actual physiological function of the human apoptosis inhibitor Nrh, also referred to as BCL2L10 or Bcl-B, remains elusive.

Although in most healthy tissues the Nrh protein is nearly undetectable, clinical studies have shown that Nrh expression is correlated with poor prognosis in breast and prostate carcinomas. We have shed light on a novel mechanism by which Nrz, the zebrafish ortholog of Nrh, was found to interact with the Ligand Binding Domain (LBD) of the Inositol-1,4,5-triphosphate receptor (IP3R) type-I Ca²⁺ channel. Indeed, the regulation of IP3Rs-mediated Ca²⁺ signaling by Nrz was shown to be critical during zebrafish embryogenesis. We used the knowledge gained with the zebrafish model to investigate Nrh function in cancer. We showed that Nrh interacts with the LBD of IP3Rs via its BH4 (Bcl-2 Homology 4) domain, which is critical to regulate intracellular Ca²⁺ trafficking and cell death. Actually, this interaction seems to be unique among the Bcl-2 family, and sets Nrh as the only Bcl-2 homolog to negatively regulate apoptosis by acting exclusively at the Endoplasmic Reticulum. Furthermore, we showed that disruption of the Nrh/IP3Rs complex primes Nrh-dependent cells to apoptotic cell death and enhances chemotherapy efficiency in breast cancer cell lines.

Lastly our results bring a new insight to the role of Nrh regarding chemotherapy resistance.

Résumé Substantiel en Français

L'Apoptose, aussi appelée « Mort Cellulaire Programmée », joue un rôle clef dans de nombreux processus biologiques, normaux et pathologiques. On distingue deux voies majoritaires de l'apoptose, la voie intrinsèque au niveau de la mitochondrie, parfois couplée à la voie extrinsèque aussi appelée « voie des récepteurs de mort », activée par des signaux extracellulaires spécifiques. Les protéines de la famille Bcl-2 sont les régulateurs principaux de l'apoptose mitochondriale. Cette famille de protéines est divisée en deux sous-groupes : les pro-apoptotiques qui vont induire la mort cellulaire, et les antiapoptotiques qui vont l'inhiber en interagissant directement avec les protéines pro-apoptotiques. Les protéines de la famille Bcl-2 peuvent réguler l'apoptose en favorisant (pro-apoptotiques) ou en inhibant indirectement (anti-apoptotiques) la perméabilité de la membrane externe mitochondriale au cytochrome C. On note que d'autres stimuli peuvent également déclencher le relargage du cytochrome C, comme un influx calcique massif depuis le réticulum endoplasmique vers la mitochondrie. Le relargage du cytochrome C dans le cytosol conduit alors à l'activation des Caspases (Cystéines-Aspartate Protéases), protéases qui sont responsables du clivage enzymatique de nombreuses protéines cellulaires induisant ainsi la mort de la cellule. Au cours de la tumorigenèse, les protéines antiapoptotiques sont fréquemment surexprimées, permettant à la cellule tumorale d'échapper à la mort cellulaire. L'importance des processus biologiques contrôlés par la famille Bcl-2 en fait une famille de protéine très étudiée. Cependant, dans le groupe des anti-apoptotiques, la fonction de certains d'entre eux reste mal connue ; le produit du gène BCL2L10/nrh fait partie de cette catégorie, bien que ce gène ait été conservé dans l'évolution, en particulier chez les vertébrés.

Chez l'Homme l'expression de *nrh* dans les tissus normaux adultes est restreinte aux ovocytes, aux ovaires et aux cellules lymphocytaires B. Chez la souris, son invalidation n'a pas donné de phénotype observable. Chez le poisson zèbre, on détecte néanmoins son expression à des stades précoces du développement. Dans ce modèle, où *nrh* est appelé *nrz*, ce gène est indispensable au développement embryonnaire, son invalidation provoquant un phénotype létal. Il a en effet été montré que la protéine Nrz est capable de réguler le trafic de calcium dans la cellule en interagissant, au niveau du réticulum endoplasmique, avec IP3R1, canal calcique sensible à l'Inositol 1,4,5-triphosphate. En agissant sur l'homéostasie calcique à une étape clef du développement, Nrz est en fait capable de contrôler la formation des complexes d'actine et de myosine. En l'absence de Nrz, ces complexes se forment prématurément ce qui bloque la progression de la gastrulation et stoppe le développement de l'embryon. Dans un contexte pathologique, chez l'Homme, l'expression du gène *nrh* est corrélée à un mauvais pronostic dans des tumeurs du sein, de la prostate, du poumon, les leucémies aigües myéloblastiques, et les syndromes myélodysplasiques. Le gène *nrh* est ainsi considéré comme un marqueur de mauvais pronostic pour ces pathologies tumorales.

Au cours d'une première étude, nous nous sommes intéressés au mécanisme impliqué dans la régulation des flux calciques par l'orthologue de Nrh chez le poisson zèbre, Nrz. Pour cela, nous avons voulu déterminer les domaines d'interaction impliqués entre Nrz et zIP3R1.

Nous avons ainsi pu montrer que Nrz interagit avec le domaine de fixation du ligand IP3 du récepteur IP3R1 (IP3BD) via son domaine BH4 en N-terminal, une caractéristique unique à ce jour parmi les protéines de la famille Bcl-2. Nous avons aussi montré que le domaine BH4 de la protéine Nrz est requis pour l'interaction avec le récepteur IP3R1, mais n'est pas suffisant à lui seul pour inhiber le relargage de calcium via IP3R1. Grâce à la présence dans les bases de données des structures tridimensionnelles des orthologues murins du domaine de liaison du ligand IP3 et de Nrh, nous avons pu modéliser l'interaction par bioinformatique et valider le modèle obtenu par la génération de mutants ponctuels du domaine de liaison du récepteur IP3R1. Nous avons pu observer que les résidus impliqués dans la fixation de l'IP3 sont différents de ceux impliqués dans la fixation de Nrz, suggérant respectivement deux sites d'interaction distincts sur le même domaine du récepteur IP3R1 pour chacune de ces molécules. Nous avons également réalisé l'étude in vitro de la fixation du ligand IP3 marqué au FITC sur le récepteur IP3R1 par polarisation de fluorescence, et nous avons pu déterminer clairement que la protéine recombinante Nrz inhibe la fixation de l'IP3 de manière dose-dépendante. Pour conclure, nous avons montré que Nrz interagit via son domaine BH4 avec le domaine IP3BD. Cette interaction permet à Nrz d'inhiber la fixation de l'IP3, ligand du récepteur IP3R1, et ainsi de réguler négativement les flux calciques.

Dans un deuxième temps, nous avons voulu déterminer la contribution de la protéine Nrh humaine dans les pathologies tumorales, à travers des propriétés similaires à son orthologue chez le poisson, Nrz. Nous avons montré l'expression de Nrh dans de nombreuses lignées cellulaires telles que les MDA-MB-231 et CAL51 issues de carcinomes mammaires, mais pas dans les MCF10A issues de tissu mammaire nontumoral. Nous avons ensuite déterminé la localisation de la protéine Nrh endogène par fractionnement subcellulaire dans les MDA-MB-231 et CAL51. Contrairement à la majorité des protéines de la famille Bcl-2 dont la localisation se répartit entre la mitochondrie et le réticulum endoplasmique, nous avons montré que la protéine Nrh est uniquement localisée au réticulum. En parallèle, nous avons montré que l'inhibition transitoire de l'expression de *nrh* par siRNA sensibilise les cellules à l'apoptose en absence de stress ou lors d'un stress calcique (thapsigargine), mais ne sensibilise pas à l'apoptose induite par la staurosporine. Nous avons également généré des mutants de Nrh ciblés soit au réticulum endoplasmique, soit à la mitochondrie et vérifié leur localisation par immunofluorescence et fractionnement subcellulaire. En traitant les cellules transfectées par ces différentes constructions à la thapsigargine pour induire un stress calcique, nous avons montré que seul l'adressage au réticulum endoplasmique est capable de protéger efficacement les cellules de l'apoptose. Nous avons observé que l'interaction est conservée entre le domaine de liaison du ligand de hIP3R1 et le domaine BH4 de Nrh. Nous avons également montré que Nrh régule négativement les flux calcique, et que son domaine BH4 est requis pour cet effet.

Nous avons obtenu des résultats démontrant qu'il est possible de moduler l'interaction de Nrh avec le récepteur IP3R1, en exprimant un peptide issu des 23 premiers acides aminés de la protéine Nrh pour rompre l'interaction entre Nrh/IP3R1 *in cellulo*. Ce peptide est notamment capable de se fixer non pas sur le récepteur IP3R1, mais sur la protéine Nrh elle-même et de supprimer ses fonctions anti-apoptotiques. Sur la base de ces données, nous avons formulé un peptide Nrh 1-23, fusionné à un domaine TAT d'internalisation, pour traiter différente lignées tumorales et tester l'effet fonctionnel de la dissociation de l'interaction Nrh/IP3R1. Nous avons montré que la dissociation de l'interaction est corrélée à l'augmentation de la sensibilité à la thapsigargin dans les MDA-MB-231. Le traitement par le peptide TAT Nrh 1-23 s'est avéré augmenter la sensibilité à la thapsigargin dans les MDA-MB-231, mais pas dans les Cellules MCF10A n'exprimant pas Nrh. Pour finir, nous avons mis en évidence que la dissociation du complexe Nrh/IP3R1 par le peptide TAT Nrh 1-23 augmente la sensibilité des cellules MDA-MB-231 et CAL51 à différentes drogues de chimiothérapie, et abolit la croissance tumorale des cellules MDA-MB-231 en agar mou.

Pour résumer, la protéine Nrh est majoritairement **réticulaire** dans les lignées modèles MDA-MB-231 et CAL51. L'interaction avec le récepteur IP3R1 humain est conservée, via les mêmes domaines que chez le poisson, et la protéine **Nrh régule négativement les flux calciques** de la même façon que Nrz. De plus, nous avons montré que la protéine Nrh endogène ou transfectée est impliquée dans la **résistance à l'apoptose** induite par les stress calciques au niveau du réticulum endoplasmique. Enfin, nous avons montré qu'il est possible de **cibler et rompre l'interaction** *in cellulo* en utilisant un peptide basé sur le domaine BH4 de Nrh.

Publications

Article 1: Bonneau B, <u>Nougarede A</u>, Prudent J, Popgeorgiev N, Peyriéras N, Rimokh R, Gillet G. The Bcl-2 homolog Nrz inhibits binding of IP3 to its receptor to control calcium signaling during zebrafish epiboly. Sci Signal. 2014 Feb 11;7(312):ra14. See pages 96 to 122

Article 2: <u>Nougarede A</u>, Popgeorgiev N, Rimokh R, Gillet G. Bcl-2 homolog BCL2L10/Nrh controls cell death and tumor progression at the Endoplasmic Reticulum. *In preparation for submission* See pages 126 to 158

Brevets

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Adrien

PREFACE

Cancer is a one of the worldwide causes of death according to the WHO. Whether it is related to environmental exposure, genetics or unknown causal effect, cancer will always rely on genetic cell alteration to promote growth and survival of some cell populations over healthy somatic cells.

Among the features of the so-called "cancer cell", Douglas Hanahan and Robert Allan Weinberg underline an exquisite intrinsic property: immortality. How could these cancer cells have mastered something human kind has been seeking after for centuries?

Fortunately we could reverse the curse, using their addiction to life at our advantage and exploit the inherent shortcomings of prolonged survival. This will allow us to take a straight shot at the Achilles' heel of cancer cells, targeting the gears that make them immortal and curing the disease. However, such a strategy requires precise understanding of how the cells are becoming "deathless" to work effectively. Along the pages of the following manuscript, we will thoroughly discuss how cells can acquire immortality using a set of proteins called the Bcl-2 family, and the means to aim at them. At last, we will review and assess the role played by one of the least understood among the Bcl-2 club, the regulator BCL2L10/Bcl-B/Nrh.

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INTRODUCTION

1. How to survive against the odds? Tales from the Bcl-2 protein family

1.1. Early work on oncogenes

At the end of the sixties it was admitted that cancer cells were carrying mutations on precise genes, conferring them a growth advantage over healthy cells. Due to the oncogenic properties of these genes, they were called **oncogenes**. These oncogenes could be endogenous, supposedly at the time from viral genome integration and vertically transmitted to their offspring, with their expression repressed under normal conditions (Huebner et Todaro, 1969). Spontaneous occurrence of cancer could be explained by genetic mutations, which will lead to derepressed oncogenes.

One source of these mutations could be environmental exposure to carcinogens, or irradiation. Using a bacterial model to test mutagenicity of compounds known to induce tumors in rodents, Mc Cann and colleagues were able to show a striking correlation between mutagenic properties and carcinogenicity (Mc Cann et al., 1975).

A well-known and direct way leading to transformation was an infection by a RNA virus like the Rous Sarcoma Virus (RSV) in fowl, the first transmissible agent ever described to induce cancer (Rous, 1911). Indeed, infection by the RSV allows a direct gene transfer of the RNA contained in the viral particles to the host DNA (Temin, 1971). Amazingly, isolation of the nucleic sequence required for neoplastic transformation showed that the sequence was also present in the normal avian DNA (Strehelin, 1976). Therefore, the coding gene for the viral sequence was called *v*-src in contrast to the normal cellular gene called *c-src* (Purchio et al., 1978; Oppermann et al., 1979; Sefton et al., 1980). It was shown that both src genes encoded for a 60kDa protein with an unprecedented enzymatic activity, the ability to phosphorylate other cellular proteins substrates on tyrosine amino-acid residues (Hunter et al., 1980). The expression of *v-src* following an infection by the RSV, through host genome integration, contributes to enhance proliferation of the infected healthy cells, and drive them into tumor cells. The idea was that the *c-src* gene might play a role in normal cells, but was tightly regulated, and that deleterious mutations might lead to *c-src* misregulation and uncontrolled proliferation (Erikson et al., 1981; Bishop, 1981). Similar discoveries occurred simultaneously with other viruses inducing tumors, such as the Avian Leukosis Virus (ALV). Upon ALV infection the *c-myc* gene, cellular counterpart of the transforming gene from the MC29 virus, was activated by the insertion of a strong viral promoter upstream of *c-mvc* coding sequence, leading to oncogenic transformation (Hayward et al., 1981; Neel et al., 1981).

1.2. Discovery of the Bcl-2 protein family

A karyotype study using a cell line established from a patient with acute lymphoblastic leukemia, was the first to show the contribution of chromosome rearrangements t(14;18) and t(8;14) to oncogenic transformation in lymphoma (Pegoraro et al., 1984). Pegoraro and colleagues showed that the first step of oncogenic transformation might be a chromosome rearrangement from the chromosome 18, containing an unknown oncogene, to the chromosome 14 where the immunoglobuline heavy chain gene was located (Pegoraro et al., 1984). A second step would be a translocation from chromosome 14 to 8, where the oncogene *c-myc* resides. The unknown oncogene carried by the chromosome 18 segment was called B cell lymphoma 2 (*bcl-2*), hence giving its name to the *bcl-2* oncogene.

The *bcl-2* gene was later cloned (Tsujimoto et al., 1985), and it was shown that the translocation t(14;18) leads to an hybrid transcript, comprising 5' sequence of bcl-2 mRNA fused with a 3' untranslated region from the immunoglobulin heavy chain (Cleary et al., 1986). This transcript still encodes for a normal Bcl-2 protein, but the added 3' region may alter *bcl-2* expression through transcriptional activation or enhanced *bcl-2* mRNA stability.

Early studies showed that Bcl-2 was a membrane protein (Tsujimoto et al., 1987). However, little was known about the function of the protein encoded by the *bcl-2* and how it could contribute with *c-myc* to oncogenic transformation. It was shown that unlike *c-myc* or *src* which promote cell proliferation, *bcl-2* was involved in survival upon growth factor deprivation, such as Interleukin 3 (IL-3) withdrawal. Expression of *bcl-2* in B-cells deprived of IL-3 allowed them to survive, but not proliferate under such conditions, highlighting the need for another oncogene like *c-myc* for complete oncogenic transformation (Vaux et al., 1988; Nunez et al., 1989). Thereby, a new class of oncogenes was defined, not as growth inducers but as promoting cell survival under adverse conditions. Precisely, Bcl-2 was shown to prevent programmed cell death, also called "apoptosis", a natural cellular process known to eliminate damaged cells and cell surplus from the body (Kerr et al., 1972; Hockenbery, 1990).

1.3. A larger family: classification of the Bcl-2 proteins

Not long after Bcl-2 discovery, numerous studies appeared to show proteins with a high sequence homology with Bcl-2 in human as well as in other species. Bcl-2 was not alone, but only the flagship of a protein family involved in the control of cell survival.

The canonical definition of a Bcl-2 protein encompasses at least one, of the four existing **BH** domain (**B**cl-2 **H**omology domain). Most of the Bcl-2 proteins also have a membrane spanning domain, which is in most cases, as for Bcl-2 itself, essential to their functions (Aouacheria et al., 2013). They are classified into three groups: multidomain anti-apoptotic, multidomain pro-apoptotic, and BH3-only proteins (See **Figure 1& Table 1**).

1.3.1. Multidomain anti-apoptotic Bcl-2 proteins

Among the multidomain anti-apoptotic group of Bcl-2 proteins, we found Bcl-2 and five related homologs: Bcl-xL (or Bcl2L1) (Boise et al., 1993), Mcl-1 (or Bcl2L3) (Kozapas et al., 1993), Bfl-1 (or Bcl2A1) (Lin et al., 1993), BCL2L10 (or Bcl2L10/Nrh/Diva/Boo) (Gillet et al., 1995) and Bcl-W (or Bcl2L2) (Gibson et al., 1996). One of the principal characteristics of these proteins is that they all have four BH domains. Interestingly, what defines the Bcl-2 multidomain class as unique is the BH4 domain, which is essential for their anti-apoptotic properties (Hanada et al., 1995). This BH4 domain is basically an amphipatic alpha-helical domain found in the N-terminal moiety of the multidomain anti-apoptotic Bcl-2 proteins. It was clearly identified using a primary sequence alignment in Bcl-2, Bcl-xL and Bcl-W, but was less conserved in Bfl-1, Mcl-1 and Bcl2L10 (Reed et al., 1996). Indeed, compared to the BH3, BH1 and BH2 domain, the BH4 domain is the less conserved of the Bcl-2 family (Huang et al., 1998; Aouacheria et al., 2005). Moreover, the loss of the BH4 domain not only impairs the anti-apoptotic function of Bcl-2 proteins, but also converts them into a pro-apopotic factor. This could happen either by alternative splicing for Bcl-xL (Bcl-xS short pro-apoptotic isoform) (Reed, 1999) and Mcl-1 (Bingle et al., 2000) or by direct proteolytic clivage for Bcl-2 (Cheng et al., 1997; Kirsch et al., 1999), Bcl-xL (Clem et al., 1998; Basanez et al., 2001), Mcl-1 (Herrant et al., 2004; Weng et al., 2005) and Bfl-1 (Kucharczak et al., 2005; Valero et al., 2012).

With the exception of Bfl-1, all multidomain anti-apoptotic Bcl-2 proteins contain a hydrophobic alphahelical C-terminal transmembrane domain (TM), allowing anchoring in organelle membranes. Without their TM, Bcl-2 proteins are soluble cytosolic proteins. To date, each Bcl-2 member has a specific subcellular localization pattern, which is a prerequisite to understand its functions into the cell (**Table** 1). While the TM domain of Bcl-2 proteins is required for membrane anchoring, sequences and mechanisms responsible for targeting specificity within the cell remain poorly defined (Schinzel et al., 2004a). Precise localization to a specific organelle might also involve other domains, as it is the case for Mcl-1 N-terminal domain (Germain et al., 2007).

Several concurring studies have shown multiple localization sites for Bcl-2 itself at the endoplasmic reticulum (ER) membrane, nuclear envelope and mitochondrial outer membrane (Krajewski et al., 1993; Lithgow et al., 1994; Haldar et al., 1994; Akao et al., 1994).

Mcl-1 subcellular localization seems to overlap with Bcl-2 for the most part, being present at the ER, nuclear and mitochondrial membranes (Yang et al., 1995). Mcl-1 was also found to be localized in the nucleus in response to DNA damage (Pawlikowska et al., 2010) and to the mitochondrial matrix where it may regulate mitochondrial respiration (Perciavalle et al., 2012).

In early studies, Bcl-xL was observed into the mitochondria, but loss of TM was not deleterious to its anti-apoptotic functions (Fang et al., 1994; Gonzalez-Garcia et al., 1994). It was found later that Bcl-xL has a dynamic subcellular localization between mitochondrial membranes and the cytosol essential to its function (Hsu et al., 1997a; Edlich et al., 2011).



Figure 1 - Topology of the Bcl-2 proteins

Figure 1 – Topology of the Bcl-2 proteins

Bcl-2 proteins are split in 3 groups from their activity toward apoptosis and the number of BH (Bcl-2 Homology) domains that they comprise: multidomain anti-apoptotics, multidomain pro-apoptotics, and BH3-only proteins. Interestingly, some proteins have a divergent BH4 or BH3 domain relative to Bcl-2 first characterized protein sequence (Aouacheria et al., 2013).

Bcl-2 homolog	ТМ	Mitochondria	Endoplasmic Reticulum	Cytosol	Others
Bcl-2	Yes	Yes	Yes	No	Nuclear membrane
Bcl-xL	Yes	Partial	No	Yes	_
McI-1	Yes	Yes	Yes	No	Nuclear membrane
Bcl-W	Yes	Yes	No	No	Nuclear membrane
Bfl-1/BCL2A1	Partial	Yes	No	No	-
BCL2L10/Nrh	Yes	Yes	Yes	No	Nucleus
Bax	Yes	Partial	Yes	Yes	-
Bak	Yes	Yes	Yes	No	-
Bok	Yes	No	Yes	No	Golgi Apparatus
Bim	Partial	Partial	No	Yes	-
Bik	Yes	Partial	Yes	No	-
Hrk	Yes	Yes	No	Yes	-
Bad	No	Partial	No	Yes	-
Bid	No	Partial	No	Yes	-
Bmf	No	Partial	No	Yes	-
Puma	No	Partial	No	Yes	-
Noxa	No	Partial	No	Yes	-

Table 1 - Subcellular localization of the Bcl-2 proteins

Table 1 – Subcellular localisation of Bcl-2 proteins

Bcl-2 proteins display distinct subcellular localization patterns. They can have a transmembrane (TM) domain, although some proteins have a hydrophobic tail linked with membrane anchoring but differing from a canonical TM (thus noted partial). Bcl-2 proteins can be present at the mitochondria, the endoplasmic reticulum, the cytosol or sometimes at other locations. They can be permanent resident or be at a compartment only under specified conditions (hence noted partial).

Bcl-W was found mainly at the mitochondria, but also at the nuclear envelope to some extent (O'Reilly et al., 2001). Although Bfl-1/Bcl2-A1 lacks a defined TM, it has been shown that its C-terminal domain is critical for anchoring at the mitochondria (Brien et al., 2009).

Finally, BCL2L10 was showed to be localized at the mitochondria and ER membranes in human oocytes (Guillemin et al., 2009), but was also detected in the nucleus of pathological embryos at the blastocyst stage (Guerin et al., 2013). Nrz, BCL2L10 zebrafish ortholog, was also found to have a dual mitochondrial and ER localization in zebrafish embryos (Popgeorgiev et al., 2011).

1.3.2. Multidomain pro-apoptotic Bcl-2 proteins

The multidomain pro-apoptotic Bcl-2 class regroups Bax (Oltvai et al., 1993), Bak (Farrow et al., 1995; Chittenden et al., 1995; Kiefer et al., 1995) and Bok (Hsu et al., 1997b; Inohara et al., 1998). These proteins were thought to contain initially BH3, BH2 and BH1 domains, but more recent studies have eluded the presence of a conserved BH4 motif (Kvansakul et al., 2008). Bax, Bak and Bok are known death inductors in cells, hence belonging to the so-called "multidomain pro-apoptotic" class. They also have a TM domain at their carboxy-terminal moiety. Multidomain pro-apoptotic Bcl-2 proteins are downstream effectors of apoptosis, thus tightly regulated by multidomain anti-apoptotic members, which keep them on check to prevent abnormal cell death.

Bax is known to be mainly cytosolic in absence of cellular stress (Hsu et al., 1997a). However, during induction of apoptosis, subcellular redistribution of Bax to the mitochondria was observed, thanks to its TM domain (Wolter et al., 1997). It was proposed that Bax reside in the cytosol with its TM unexposed at steady-state, but undergoes conformational changes upon cellular stress and relocates to the mitochondria (Schinzel et al., 2004b).

A recent study has shown that Bax is constitutively present at the mitochondria, but is retrotranslocated to the cytosol by Bcl-xL in absence of stress signals (Edlich et al., 2011). This retrotranslocation was later observed independent of Bcl-xL or other Bcl-2 multidomain anti-apoptotic proteins. In another study, Bax mitochondrial dissociation rate was found inversely correlated with the priming to apoptosis in human cells (Schellenberg et al., 2013).

Bax can be localized at the ER at steady-state, and can induce cell death from this organelle following a death signal (Zhong et al., 2003). Besides, Bax translocation to the ER but not the mitochondria was observed after STAT1 activation, triggering cell death (Stout et al., 2007).

In early studies, Bak was found at the ER membrane and at the mitochondria outer membrane (Zhong et al., 2003). However, unlike Bax, Bak is permanently present at the mitochondrial outer membrane. Indeed, Bak localization to the mitochondria relies on its interaction with VDAC2, a mammalian specific isoform of the Voltage-Dependent Anion Channel (Cheng et al., 2003). Through this interaction, Bak is bound to the mitochondria, but in an inactive form. Subsequent release by stress signal are a necessary trigger to Bak-mediated cell death at the mitochondria (Ren et al., 2009; Leshchiner et al., 2013).

Unlike Bax and Bak, which are widely expressed among many tissues, Bok expression seems restricted to reproductive organs (Hsu et al., 1997b). Bok is mainly localized at the ER membrane and the Golgi apparatus, but was observed to some extent in mitochondrial and nuclear fractions (Echeverry et al., 2013).

1.3.3. BH3-only pro-apoptotic Bcl-2 proteins

This class regroups the most numerous members, which contain only one BH3 domain defining their function. To date, there is eight canonical members of this class, though more and more proteins are found comprising a BH3 domain: Bad (Yang et al., 1995), Bik (Boyd et al., 1995), Bid (Wang et al., 1996), Hrk (Inohara et al., 1997) Bim (O'Connor et al., 1998), Noxa (Oda et al., 2000), Puma (Nakano et Vousden, 2001; Yu et al., 2001) and Bmf (Puthalakath et al., 2001). The BH3-only proteins are known as modulators of apoptosis, either by activating multidomain pro-apoptotic, or inhibiting multidomain pro-apoptotic Bcl-2 members. BH3-only proteins are regulated through a myriad of cellular signaling pathways, engaging them when death is required, and disabling them when proliferation or survival is required.

Among the BH3-only proteins, all but Bik and Hrk lack a transmembrane domain (Lemonosova et al., 2008). As a result, most BH3-only protein are residing in cytosol, but are relocated upon cellular stress to exert their functions, mainly at the mitochondria.

Bad was shown to be sequestered to the cytosol upon phosphorylation by Akt on one of its serine residues, bound to a protein called 14-3-3 (Zha et al., 1996; Datta et al., 1997).

Bid is resident to the cytosol in an inactive Full-length form (Wang et al., 1996). Upon death stimulation, Bid is cleaved, resulting in the translocation of tBID (namely truncated BID) to the mitochondria, engaging Bax/Bak dependent apoptosis (Li et al., 1998).

Bim and Bmf are mainly cytosolic and associated respectively with microtubule network and actin filament network (Puthalakath et al., 1999; Puthalakath et al., 2001). In response to cytoskeleton disorganization they are released to activate anoikis, a specialized form of apoptosis occurring when the cell detaches from the surrounding extracellular matrix (Martin et Vuori, 2004). Bim has no transmembrane domain but displays a hydrophobic region at its C-terminal tail with similar functions (Lemonosova et al., 2008).

Puma and Noxa are transcriptionally regulated by the tumor suppressor p53. They are part of p53 program to eliminate abnormal cells and prevent oncogenic transformation. Upon p53 induction, Puma and Noxa are expressed and trigger cell death at the mitochondria (Shibue et al., 2003; Yakovlev et al., 2004). Bik and Hrk, containing a C-terminal TM domain, are membrane-targeted proteins. While Hrk seems mainly present at the mitochondria (Sunayama et al., 2004), Bik seems present for the most part at the ER membrane (Germain et al., 2002), although a mitochondrial fraction was also observed (Han et al., 1996; Hedge et al., 1998).

1.4. Live and let die: Involvement of the Bcl-2 family in cellular programs mediating death or survival

The Bcl-2 proteins are master regulators of life and death of the cell. Cell death can be either controlled and active, or passive. Therefore, a distinction is made between active programs controling cell death or cell survival, and passive death commonly called Necrosis. So far, several programs regulating cell life and death have been defined, such as Apoptosis, Autophagy, or Necroptosis. In the following part, we will mainly discuss the involvement of Bcl-2 proteins in Apoptosis and Autophagy, as there has not been any evidence so far of direct or indirect role played by Bcl-2 proteins in Necroptosis.

1.4.1. Apoptosis: A matter of honour

In ancient Japan, a defeated samurai warrior would commit a ritual suicide called *seppuku* to avoid the burden and shame of defeat, following the *bushido* code.

Apoptosis could similarly be associated in a way of self-killing to prevent the release of immunogenic molecules that could otherwise trigger an immune response. This process offers a clever physiological way of clearing an excess of cells in multicellular organisms, contributing to the balance between cell proliferation and cell death. Supernumerary cells can result from developmental processes, or cell with genetic abnormalities. Hence, regulation of apoptosis is a critical cornerstone in diseases, as a lack of apoptosis might trigger cancer (Hanahan et Weinberg, 2011), and an excess neurodegenerative disorders (Friedlander, 2003).

Three steps are distinguished in apoptosis: (1) Initiation, (2) Regulation and (3) Execution.

During the initiation step (1), the cell is perceiving physiological or pathological danger signals through an array of sensor proteins. The regulation phase (2) relies heavily on the Bcl-2 protein family to integrate multiple cellular signals and initiate the commitment to cell death. Once the barrier is crossed, the decision is irreversible and cell fate sealed. The last stage (3) involves execution of apoptosis by the Caspases (Cystein-Aspartate proteases), neutralizing many cellular proteins by direct cleavage, as well as molecules with immunogenic properties.

A distinction is made between extrinsic apoptosis pathway, which is triggered by the binding of an extracellular ligand to a receptor on the plasma membrane, and the mitochondrial pathway (or intrinsic pathway) which hinges upon mitochondria to integrate intracellular danger signals and execute apoptosis (Fulda et Debatin, 2006).

Therefore, caspases are considered the hallmark of apoptosis, as they are the downstream converging effectors of any apoptotic cell death pathway.

1.4.1.1. Caspases: the katana sword

Caspases are enzymes that were first identified as orthologs to the *ced-3* gene in *C.Elegans*, an apoptosisinducing gene with homology in sequence and biological activity to the mammalian Interleukin-1- β -Converting Enzyme (ICE) (Miura et al., 1993; Yuan et al., 1993). Caspases have a conserved structure, with an N-terminal pro-domain, a central large catalytic domain and C-terminal small conserved motif (See **Figure 2**). They are produced in an inactive precursor form, and need to dimerize in order to be active. Dimerization is typically facilitated by a direct interaction with helper proteins, and can necessitate N-terminal cleavage of the pro-caspase (Fuente-Prior et Salvesen, 2004; McIlwain et al., 2013). Caspases activation in the cell is a multistep process that requires at first activation of initiator caspases. These initiator caspases subsequently activate the effectors caspases, which cleave a broad range of cellular substrates.

There are fourteen identified mammalian caspases to date, divided into three groups: Initiator Caspases (Caspase-2, 8, 9, 10 and 12), Effector Caspases (Caspase-3, 6 and 7), and finally Inflammation-related Caspases (Caspase-1, 4, 5). Caspase-11 is an inflammation-related caspase present in mouse but not in human, with a function similar to human caspase-4 (Shi et al., 2014). Caspase-13 was initially thought to be another human caspase ortholog but turned out to be the bovine ortholog of caspase-4 (Koenig et al., 2001). Caspase-14 is the most divergent caspase to date, and unlike the widespread expression of most caspases is only found in the epidermis, where it might play a role in skin homeostasis and disease (Denecker et al., 2008).

1.4.1.1.1. Initiator Caspases

Initiator capases are the first to be activated upon commitment to apoptosis. They are characterized by a longer N-terminal domain containing different protein-protein interaction domains. Initiator caspases-2 and 9 are characterized by a CARD domain (Caspase Recruitement Domain), whereas caspases-8 and 10 contain a DED domain (Death Effector Domain). These domains lead to the formation of protein complexes with caspase adapter proteins, creating activation platforms for the caspases. Indeed, activation of caspase-8/10, 2 and 9 does not require cleavage, but dimerization with the help of other proteins within a protein complex, triggered by upstream signaling events (McIlwain et al., 2013; Parrish et al., 2013). Besides, dimerization promotes autocatalytic cleavage of the N-terminal CARD-containing or DED-containing domain in capases mononers, further enhancing the stability of the dimer.

Caspase-12 could represent a class of itself, as it has been classified both as an initiator caspase (containing a DED domain) and an inflammation-related caspase. Nevertheless, caspase-12 seems to be a specialized initiator caspase, resident at the Endoplasmic Reticulum (ER), and able to trigger apoptosis in response to specific ER stresses (Nakagawa et al., 2000; Saleh et al., 2006; McIlwain et al., 2013).



Small subunit

DED-domain CARD-domain

Large catalytic subunit

Figure 2 - Domain structure of human caspases

Figure 2 – Domain structure of human caspases

Human caspases are divided into 3 groups: apoptosis initiator caspases, apoptosis executioner caspases and inflammation caspases. They share a small C-terminal domain and a large central catalytic domain. They can display at their N-terminal moeity a DED or CARD domain allowing protein-protein interactions required for their recruitment and activation.

1.4.1.1.2. Effector Caspases

Effector caspases-3, 6 and 7 are the molecular scissors supervising the dismantlement of vital cell components during apoptosis. Indeed, caspase-3 is responsible for the bulk of proteolysis while caspase-6 and 7 appear to have a somewhat minor role, and exhibit a different substrate specificity from caspase-7 (Walsh et al., 2008). To this date, approximately 520 proteins are known and experimentally validated as caspases substrates (Lüthi et Martin, 2007). Caspase cleavage does not always eliminate the biological function of its targets. For example, caspase-3 mediated cleavage of the endonuclease ICAD leads to a truncated form of the protein, CAD, responsible for DNA degradation upon apoptosis induction, a well-known criterion to identify apoptotic cell death (Sakahira et al., 1998).

Effector caspases-3, 6 and 7 do not contain oligomerization domain such as CARD or DED domains, therefore are only activated through direct proteolytic cleavage by initiator caspases (Pop et Salvesen, 2009). Finally, effector caspases can also behave as amplifier of the apoptotic cascade, activating by cleavage upstream caspases.

1.4.1.2. Extrinsic Regulation of Apoptosis

The extrinsic pathway of apoptosis, often called death receptors pathway, is required for instance to induce cell death in aberrant immune cells (T lymphocytes) that could otherwise do harm to tissues through inflammatory response or overproliferate leading to lymphoma. The extrinsic pathway of apoptosis delivers cell death from ligand binding signals at the plasma membrane (See **Figure 3**). The receptors involved are members of the TNF-receptor superfamily. The CD95 (also called APO-1/Fas) and TRAIL receptors (TNF-Related Apoptosis Ligand) activation are mainly involved in apoptosis, whereas other members of the TNF receptors family may instead trigger the transcription of target genes linked with a pro-inflammatory response (Fulda et Debatin, 2006).

All death receptors share a common topology, with an extracellular domain containing between 2 to 4 cystein-rich repeats involved in ligand binding, and a specific intracellular domain to recruit partner proteins. This intracellular domain makes for the specificity of signaling output upon binding of TNF-related ligand, whether it is directly inducing apoptosis of gene transcription (Walczak, 2013). In the case of CD95 and TRAIL, the intracellular domain is called Death Domain (DD) from its ability to control apoptosis execution.

The binding of their respective ligands induce the trimerization of CD95 and TRAIL receptors, with the subsequent recruitment of FADD (Fas-Associated Death Domain) through their DDs.



Figure 3 - Extrinsic Pathway of Apoptosis

Figure 3 – Extrinsic Pathway of Apoptosis

Upon respective ligand binding on CD95 or TRAIL death receptor, trimerisation occurs leading to the recruitment of FADD via their Death Domain. FADD can either recruit caspase-8 or 10 through its own DED domain and activate them. Caspase-8/10 can directly activate executioner caspase-3 or trigger cytochrome c release through tBid-mediated Mitochondrial Outer Membrane Permeabilization (MOMP). Cytochrome c triggers the assembly of the Apoptosome, the activating plateform for caspase-9, which will in turn activate caspase-3, engaging the later stage of apoptosis.

As FADD contains a DED domain, it is able to recruit caspase-8 and caspase-10. The complex holding a CD95 or TRAIL trimer, FADD and caspase-8/10 is hence called DISC (Death-Inducing Signaling Complex). Through caspase-8/10 activation, the DISC complex executes apoptosis downstream events, such as activation of the executioner caspase-3.

Although the basic process of caspases activation by the death receptors pathway is independent of any regulation by the Bcl-2 family, they play a key role in regulating the amplification and overall apoptotic response (Kuwana et al., 1998).

In fact, the activated caspase-8/10 can also cleave the BH3-only BID into its truncated form tBID (Li et al., 1998; Luo et al., 1998). This truncation exposes a glycin residue, which undergoes N-myristoylation, allowing its translocation at the mitochondria, where tBID is responsible for Bak and Bax activation, leading to the mitochondrial way of apoptosis (Zha et al., 2000). Therefore, tBID represents the connection between the extrinsic and intrinsic pathways of apoptosis.

1.4.1.3. Mitochondrial Regulation of Apoptosis

The intrinsic pathway of apoptosis takes place at the mitochondria, the cell "nuclear reactor", which produce most of the cellular energy from the oxidative phosphorylation.

Mitochondrial apoptosis can be activated through a plethora of cellular danger signals, such as DNAdamage, ionizing radiations, mitotic failure or privation from growth factors. When the time comes to execute mitochondrial apoptosis, the effector proteins Bax and Bak are activated to punch holes through the Outer Mitochondrial Membrane (OMM). This process is the keystone of mitochondrial apoptosis, and is called mitochondrial outer-membrane permeabilisation (MOMP). MOMP can also result from the opening of a protein pore complex called mPTP (mitochondrial Permeability Transition Pore). While the Bax/Bak-dependent MOMP is tightly controlled by the Bcl-2 proteins, mPTP opening is dependent on mitochondrial calcium elevation (Baumgartner et al., 2009). In both cases, MOMP induces the release of pro-apoptotic factors from the mitochondria Intermembrane Space (IMS) to the cytosol (See **Figure 4**).

1.4.1.3.1. Mitochondrial-released pro-apoptotic factors

The main pro-apoptotic factor released from mitochondria is a 13 kDa positively-charged haemcontaining protein, named cytochrome c. Cytochrome c release is considered the hallmark of apoptosis, a point of no return from which cell death is thought irreversible.

Although encoded by a nuclear gene, cytochrome c is residing at the mitochondria, more precisely in the Inter Membrane Space (IMS), within involuted cavities formed by the Inner Mitochondrial Membrane (IMM) called cristaes (Ow et al., 2008). Cytochrome c is bound at the IMM through its interaction with cardiolipin, a negatively-charged phospholipid specific to the mitochondria.

Using its haem group, cytochrome c is able to shuttle electrons between Complex III and Complex IV of the respiratory chain, thus participating in the mitochondrial respiration under normal conditions.

Involvement of cytochrome c in apoptosis, either using *in vitro* cell-free system or *in vivo*, has long been established (Liu et al., 1996; Kluck et al., 1997; Brustugun et al., 1998). Under apoptotic conditions, cytochrome c release from mitochondria occurs as a multistep process.

The first step requires the mobilization of cytochrome c stores and breakdown of its association with cardiolipin. The affinity between cardiolipin and cytochrome c is lowered upon oxydation of cardiolipin, through the action of Reactive Oxygen Species (ROS), as well as phospolipase A2. Calcium is also thought to induce the detachment of cytochrome c from cardiolipin by weakening the electrostatic interaction between the two molecules (Ow et al., 2008).

In a second step, as the OMM is totally impermeable to molecules as large as cytochrome c, the mitochondrial integrity needs to be physically breached in order to release cytochrome c into the cytosol. This step involve either Bax/Bak proteins, or the opening of mPTP as previously mentioned. Once released, cytochrome c binds to the apoptotic protease-activating factor-1 (Apaf-1) in the cytosol (Zou et al., 1997; Li et al., 1997). Apaf-1 is a cytosolic protein containing a CARD domain, present as a monomer at steady-state, but oligomerizes in a cytochrome c and ATP -dependent fashion. The assembly of seven Apaf-1 monomer into a wheel-like structure is able to recruit and activate procaspase-9, forming a unique complex called "apoptosome" (Acehan et al., 2002). Activated caspase-9 therefore triggers downstream events of the apoptotic cascade such as caspase-3 activation and caspase-3 – mediated proteolysis. Besides, some Bcl-2 proteins have been reported to directly bind and neutralize cytosolic cytochrome c, such as Nr-13, chicken ortholog of BCL2L10/Nrh (Moradi-Ameli et al., 2002). Furthermore, cytochrome c has been shown to interact with inositol - 1,4,5 – triphosphate receptors (IP3Rs) and promotes sustained calcium transient during apoptosis, further enhancing the apoptotic response (Boehning et al., 2003).

In addition to cytochrome c, other apoptotic factors have also been reported to be released from the mitochondria during apoptosis. The Apoptotic-Inducing Factor (AIF) protein is a protease encoded by a nuclear gene but present in the IMS. Once released in the cytosol, AIF can directly trigger caspase-3 activation but also migrate in the nucleus inducing DNA fragmentation (Susin et al., 1997). However, AIF-mediated DNA fragmentation seemed rather indirect and might require other intermediates, such as CAD or Endonuclease G (EndoG) (Wang et al., 2002).

EndoG is a mitochondrial resident nuclease released during apoptosis, which is linked with chromatin DNA cleavage into nucleosomal fragments (Li et al., 2001; Parrish et al., 2001). Of note, EndoG activity seems independent of caspase activation.



Figure 4 - Mitochondrial Pathway of Apoptosis

Figure 4 – Mitochondrial Pathway of Apoptosis

Upstream Bax/Bak activation triggers Mitochondrial Outer Membrane Permeabilization (MOMP) and the subsequent release of multiple pro-apoptotic factors. Among them, cytochrome c is the one with the most dramatic effect, as it is promoting the Apoptosome assembly. The Apoptosome complex is a formed with seven Apaf-1, pro-caspase-9 and cytochrome c molecules, leading to caspase-9 activation. Activated caspase-9 is directly responsible for downstream executioner caspase-3. Both caspase-9 and 3 activity could be suppressed by Inhibitor of Apoptosis Proteins (IAPs). The IAPs can be inhibited by MOMP-released Smac/DIABLO and Htra2/Omi. Finally, MOMP-released AIF and EndoG are responsible for apoptotic DNA degradation independently of caspases.

Finally, the last class of mitochondria released pro-apoptotic factors are represented by Smac/DIABLO (Verhagen et al., 2000; Du et al., 2000) and Htra2/Omi (Suzuki et al., 2001). These proteins are antagonists of Inhibitors of Apoptosis (IAPs) proteins. The IAPs family proteins, which encompass eight members in mammals, are known to bind directly and inhibit both initiators and effector caspases (Srinivasula et Ashwell, 2008; Fulda et Vuccic, 2012).

Although both Smac/DIABLO and Htra2/Omi are able to bind IAPs, they seemed to differ in the mechanism involved in the IAPs inhibition. Smac/DIABLO binding might be sufficient to inhibit IAPs, whereas Htra2/Omi requires its serine protease activity to cleave and inactivate IAPs (Verhagen et al., 2002).

1.4.1.3.2. The Bax/Bak-dependent apoptosis

At steady-state, Bax and Bak pro-apoptotic proteins are kept on check by Bcl-2 anti-apoptotic proteins. They are bound through the interaction between their BH3 and the hydrophobic pocket, comprising the BH3, BH2 and BH1 domains, of anti-apoptotic proteins. As apoptosis is triggered, Bax and Bak are released from their respective neutralizing partners to execute programmed cell death (Czabotar et al., 2014). The way of releasing Bax and Bak to execute freely apoptosis involve: (a) displacement of the equilibrium between bound/inactive state and free Bax/Bak (b) Bax/Bak conformational activation by BH3-only proteins. However, this cascade of events and their relative importance to each other has long been a matter of debate in the field of apoptosis. Thereby we will review in this manuscript the **unified model** proposed by David Andrews and its colleagues, integrating elements from both (a) and (b) into a single comprehensive model (Shamas-Din et al., 2013).

1.4.1.3.2.1. A unified model for Bax/Bak activation

From the BH3-only proteins, two classes can be distinguished: BH3-only activators, which can directly activate Bax/Bak, and BH3-only sensitizers which are inhibitors of anti-apoptotic Bcl-2 proteins, thus increasing probability of the apoptotic outcome.

The BH3-only activators are represented by Bid, Bim and Puma (Letai et al., 2002; Edwards et al., 2013) whereas the remaining BH3-only are classified as sensitizers: Bad, Bik, Bmf, Noxa, Hrk. Hereby, we have a four component system where (1) BH3-only sensitizers inhibit (2) Bcl-2 anti-apoptotic proteins, which in turn inhibit (3) BH3-only activators and (4) Bax/Bak (See **Figure 5**). BH3-only activators can directly activate Bax/Bak, but a distinction is made between the modes of regulation, between BH3-only activators and Bax/Bak by Bcl-2 anti-apoptotic proteins. Indeed, Bcl-2 anti-apoptotic proteins seem more likely to regulate negatively Bax/Bak rather than BH3-only activators, although both mechanisms are known to coexist (Shamas-Din et al., 2013).



Figure 5 - Bax/Bak Activation Unified Model

Figure 5 – Bax/Bak Activation Unified Model

A four component system tightly regulating Bax/Bak activation: (1) BH3-only sensitizers inhibit (2) Bcl-2 anti-apoptotic proteins, which in turn inhibit (3) BH3-only activators and (4) Bax/Bak. To note, anti-apoptotic Bcl-2 proteins are more efficient to inhibit Bax/Bak than BH3-only activator proteins.

As a consequence, Bax/Bak-mediated apoptosis is governed by an equilibrium set between different classes of effectors explained above, depending on relative protein concentrations and binding affinities. This theory formulated at first by Korsmeyer and his colleagues has been validated since, opening the gates for pharmacological modulation of apoptosis (Korsmeyer et al., 1993; Letai, 2005).

Finally, the table of interactions provided by Rautureau and his colleagues allows an application of the unified model, integrating binding specificity among Bcl-2 proteins (Rautureau et al., 2012) (See Figure 6).

1.4.1.3.2.2. Bax/Bak-mediated pore formation

Once activated, Bax and Bak are able to perforate the OMM, releasing pro-apoptotic factors such as cytochrome c and IAP inhibitors. Indeed, Bax and Bak are required proteins for mitochondrial apoptosis, as their absence results in a severe dysfunction of mitochondrial apoptosis execution (Lindsten et al., 2000; Wei et al., 2001). Activation of Bax/Bak induces conformational changes, allowing activated Bax/Bak to oligomerize at the OMM (Westphal et al., 2014). Oligomerization of Bax/Bak at the OMM is a key feature of the MOMP, as Bax/Bak oligomerization deficient mutants, containing mutations either in their BH3 domain or their hydrophobic groove (BH1), fail to induce MOMP (George et al., 2007; Dewson et al., 2008).

The BH3 domain from a monomeric Bax/Bak associates with the helix alpha 4 and 5 from the hydrophobic groove (BH1) to oligomerize. Bax/Bak oligomer can also multimerize through an alpha 6/ alpha 6 helix interaction (Dewson et al., 2012). Furthermore, Bax/Bak homo-oligomer, as well as hetero-oligomer have been reported, hence showing a common mechanism of oligomerization between both proteins.

Besides, mitochondrial fusion/fission dynamics regulator Drp-1, as well as the OMM sphingolipid composition have been shown to modulate Bax/Bak insertion into the outer mitochondrial membrane and cytochrome c release (Montessuit et al., 2010; Chipuk et al., 2012). Recent studies have also shown that Bax-induced MOMP is dependent of the mitochondrial shape and size, underlying an effect of the OMM curvature for a correct insertion of Bax (Renault et al., 2015).

The pore resulting from Bax/Bak oligomers have been shown to involve both lipidic and proteic components (Luna-Vargas et Chipuk, 2016). Indeed, cooperation between Bax/Bak and mitochondrial lipids to induce MOMP is known, but the exact nature of the pore is still not completely understood (Kuwana et al., 2002; Qian et al., 2008; Schafer et al., 2009). From the Bax/Bak-induced pores, small molecules like the cytochrome c are able to diffuse, but also bulkier proteins, as the Bax/Bak pore display a tunable size depending on protein concentration (Bleicken et al., 2013).





Figure 6 - Interaction profile of Bcl-2 proteins

Figure 6 – Interaction profile of Bcl-2 proteins

Bcl-2 proteins are defined by interaction with each other. The interaction profiles between pro-survival and pro-apoptotic displayed above reflect strong affinity interaction only with an affinity constant (Kd) \leq 100 nM. BH3-only proteins are identified as Activators: Bim, Bid, Puma (Pale brown) or Sensitizers: Bmf, Bad, Bik, Hrk, Noxa (Green).

1.4.1.3.3. The calcium-dependent apoptosis

1.4.1.3.3.1. mPTP-induced MOMP

Mitochondrial outer membrane permeabilisation can also occur independently of the Bax/Bak pore, through the opening of a proteic pore complex called mitochondrial Permeability Transition Pore (mPTP). This protein complex, present at the inner mitochondrial membrane, will allow at open state ions and solutes up to 1500 Daltons to freely diffuse across the IMM (Bernardi, 2013).

Accumulation of calcium ions into the mitochondrial matrix is known to trigger mPTP opening and sustain its open conformation. Breaking electrochemical gradient of proton ions (H+) between the mitochondrial matrix and the inter membrane space upon mPTP opening leads to mitochondrial depolarization and the uncoupling of mitochondrial respiration (Loew et al., 1994; Bonora et Pinton, 2014). Opening the mPTP induces an osmotic matrix swelling by the influx of water, profoundly remodeling mitochondrial structures such as cristaes, which contain the main cytochrome c stockpiles. Eventually, mitochondria became swollen to the point of OMM rupture, destroying the organelle as well as releasing cytochrome c into the cytosol (Kroemer et al., 2007).

In summary, mPTP-dependent MOMP is executed in the three following steps: (1) calcium entry into the mitochondrial matrix, hence through both the OMM and the IMM (2) mPTP opening and mitochondrial depolarization (3) mitochondrial swelling, OMM rupture and cytochrome c release (See **Figure 7**).

Despite the phenomenon of mitochondrial permeability transition has been reported some time ago, the precise nature of the mPTP complex is still not completely understood (Zoratti et Szabo, 1995). However, a growing body of evidence suggest that the mPTP complex might be formed by a dimer of the F0/F1 ATP synthase (Giorgio et al., 2013). A regulatory protein called Cyclophilin D, which is the only confirmed activator of the mPTP, is also reported to modulate F0/F1 ATP synthase activity (Giorgio et al., 2009; Javadov and Kuznetsov, 2013).

In most observations, a clear link has been established between mitochondrial calcium overload and mPTP opening (Gunter et Pfeiffer, 1990; Zoratti et Szabo, 1995). Indeed, the mitochondria has a prominent role to buffer cellular calcium concentration, while keeping enough of these ions to allow a constant metabolic rate of the oxidative phosphorylation and the citric acid (TCA) cycle. It is to note that the activity of the pyruvate dehydrogenase, α -ketoglutarate dehydrogenase and isocitrate dehydrogenase, which are critical TCA cycle enzymes, is tightly regulated by calcium ions (Wan et al., 1989). Moreover, calcium spikes have been observed in concert with mitochondrial metabolic activity (Hajnóczky et al., 1995; Robb-Gaspers et al., 1998). The tight coupling between calcium spikes and metabolic activity is permitted by the close proximity of the Endoplasmic Reticulum (ER), the central reservoir of cell calcium, and mitochondria at contact sites called MAM, standing for Mitochondrial-Associated Membranes (Shore et Tata, 1977; Csordás et al., 2006).


Figure 7 - Calcium-driven apoptosis

Figure 7 - Calcium-driven apoptosis

Cytosolic calcium elevation can lead to three events converging in the induction of Mitochondrial Outer Membrane Permeabilization (MOMP) and apoptosis. (A) Cytosolic calcium increase can trigger activation of calcium-dependent proteases, called calpains. Calpains have many substrates, among them featuring Bid, Bcl-xL or Bfl-1. Calpains can activate Bid into tBid, whereas cleavage of Bcl-xL and Bfl-1 confer them pro-apoptotic properties, triggering MOMP. (B) Cytosolic calcium can also activate the calcium-dependent phosphatase calcineurin, which can dephosphorylate Bad into its active form to induce MOMP. (C) Finally, excessive mitochondrial calcium uptake through VDAC and MCU channels can lead to mitochondrial calcium overload, hence promoting mitochondrial permeability transition pore opening (mPTP). Retrograde flux of water would eventually trigger mitochondrial burst through osmotic shock, hence MOMP and apoptosis.

MAM are specialized domains enriched in transmembrane proteins such as PACS-2, Mitofusin-2 (MFN2), or Drp-1, tethering together the ER and the mitochondria (Simmen et al., 2005; de Brito et Scorrano, 2008; Prudent et al., 2015). MAM also feature 1,4,5 Inositol tri-Phosphate Receptor (IP3R) calcium channel at the ER side as well as the Voltage-Dependent Anion Channel (VDAC) at the mitochondrial side, tethered together by the Grp75 protein (Szabadkai et al., 2006; Hayashi et al., 2009). While IP3R receptors are involved in calcium release from the ER, VDAC channels are critical for mitochondrial calcium intake and transport through the OMM (Gincel et al., 2001; Rapizzi et al., 2002; Mikoshiba, 2007; Tan et Colombini, 2009).

At the contact points between ER and mitochondria, calcium concentrations can rise up to the range of 10μ M, a hundredfold higher than typical cytosolic calcium concentrations (Csordás et al., 2010). Indeed, refilling the mitochondrial matrix with calcium is made possible by an active carrier called Mitochondrial Calcium Uniporter (MCU), whose ability to transport calcium ions is severely limited at low concentration (de Stefani et al., 2011; Baughman et al., 2011). This is due to the binding of two regulatory proteins on MCU, namely MICU2 which inhibits MCU opening at low calcium concentration, and MICU1 which promotes MCU opening at high calcium concentration (Patron et al., 2014).

Thanks to the high concentration calcium microdomains at the MAM, calcium entry is efficient and unhindered through both VDAC channel at the OMM and MCU at the IMM (Giorgi et al., 2009). Besides MCU, calcium transport through the IMM can happen through a Ca^{2+/}H⁺ exchanger called Letm1 (Jiang et al., 2009; Jiang et al., 2013; Nowikovsky et Bernardi, 2014). However, because calcium influx is dependent of the electrochemical H⁺ gradient created by functional mitochondrias, Letm1 seems unlikely to trigger a massive mitochondrial calcium overload, as mitochondrial depolarization would shut down this channel (Santo-Domingo et Demaurex, 2010).

Calcium influx through the IMM is mainly due to MCU and Letm1, whereas a Na⁺/Ca²⁺ transporter called NCLX is responsible for matrix calcium extrusion (Palty et al., 2010). Under pathological conditions, an equilibrium between calcium entry and calcium efflux can be reached to some extent before mPTP opening. Upon an activating phosphorylation by PKA, NCLX has been reported to prevent calcium overload and mPTP opening by extruding calcium out of mitochondria, therefore shifting the balance toward cell survival (Kostic et al., 2015).

Furthermore, transient mPTP opening has been proposed to be a mechanism of mitochondrial calcium release, and a way to attenuate matrix calcium overload (Bernardi et von Stockum, 2012). This idea is supported by observations of transient mPTP opening without cytochrome c release or cell death. However, mPTP repetitive gating/opening cycles may still cause mitochondrial membrane potential to fluctuate (Hüser et Blatter, 1999; Petronilli, 2001). Eventually, transient mPTP-mediated calcium release might play a relevant physiological role.

Indeed, transient mPTP opening was shown to finely tune matrix calcium concentration to match metabolic demand in the heart, and shield neurons from matrix calcium overload in the brain (Elrod et al., 2010; Barsukova et al., 2011; Kwong et Molkentin, 2015).

As the whole process of mPTP-mediated MOMP is under the tight control of calcium entry in the mitochondria matrix, a prominent role is played by calcium channels at the ER, especially the IP3Rs. At last, mPTP-mediated MOMP is driven by IP3Rs calcium signals, hence underlying the contribution of IP3Rs signaling to apoptosis (Hanson et al., 2004). In fact, IP3Rs invalidation has been reported to markedly inhibit apoptosis in T lymphocytes upon T Cell Receptor (TCR) activation (Jayaraman et al., 1995; Jayaraman et Marks, 1997), but also in B cells (Sugawara et al., 1997), as well as in neurons (Blackshaw et al., 2000).

There is no evidence of a direct modulation of the mPTP by the Bcl-2 proteins, although they are heavily involved in the mPTP-triggered MOMP through regulation of calcium efflux from the ER as well as calcium influx at the mitochondria. This part will be developed in the chapter 2.

1.4.1.3.3.2. mPTP-independent apoptosis

We have previously discussed the critical role of calcium transfer from ER to mitochondria, opening the mPTP and promoting cytochrome c release. Nevertheless, cytosolic calcium elevation can also trigger Bax/Bak-dependent apoptosis, by activating calcium-dependent cysteine proteases called calpains (Blanchard et al., 1997) (See **Figure 6**).

There are twelve different calpains, but the major representatives are the m- and μ -calpain, respectively activated by calcium concentration ranging from 200 to 1000 μ M for the m-calpain and 5 to 50 μ M for the μ -calpain (Korshid et Ikura, 2002). Deregulation of calcium levels and aberrant calpain activation has been observed and linked to tissue damage in heart and brain ischemia, but also in neurodegenerative disorders (Moldoveanu et al., 2002; Goll et al., 2003).

Among the targets of calpains, there are numerous Bcl-2 family proteins, such as Bid, which is cleaved in t-Bid by calpains during myocardial ischemia/reperfusion (Chen et al., 2001). Anti-apoptotic Bcl-2, Bcl-xL and Bfl-1 are also cleaved by calpains, converting these proteins into pro-apoptotic molecules (Gil-Parrado et al., 2002; Ofengeim et al., 2012; Valero et al., 2012). Bax cleavage by calpains results in an even more potent cell death inducer (Wood et al., 1998; Cao et al., 2003). Calpains have also been reported to activate by proteolytic cleavage caspase-12, the only ER-resident caspase (Nakagawa et al., 2000; Yoneda et al., 2001). Activated caspase-12 therefore behaves as an initiator caspase and triggers downstream events of apoptosis.

Finally, Akt-mediated inhibitory phosphorylation and subsequent 14-3-3 sequestration of pro-apoptotic BH3-only protein Bad can be reversed by the calcium-dependent phosphatase Calcineurin (PP2B). Desphosphorylated and unbound Bad protein can then freely interact with Bcl-xL, inhibiting its anti-apoptotic properties and enhancing the apoptotic response (Wang et al., 1999).

1.4.2. Autophagy: the art of self-cannibalism

The ancient symbol of the *Ouroboros*, named after the greek *tail-devouring snake*, perfectly represents autophagy. Autophagy involves a process, by which cells are able to deal with cellular and organellar damages, using their capacity to recycle worn out parts, broken into the smallest possible units (i.e. amino acids) and melt them to create brand new replacement proteins. As there are several pathways of autophagy, we will refer in the present manuscript to the Macroautophagy process as Autophagy. Autophagy should not be considered as a cell death pathway *per se*, but rather a way to help commit to the decision of cell doom or cell survival, at the crossroad between life and death.

From the first discoveries, autophagy has been characterized as a programmed way of cell death. However, new concepts have recently emerged defining autophagy to be more likely a bystander of cell death instead of being the executioner. Therefore, we will discuss in the part below about the entanglement between autophagy and apoptotic cell death, and the part played by the Bcl-2 proteins.

1.4.2.1. Autophagosome formation

Autophagy is essential both for promoting survival, making available new amino acids from the cell own components, and assisting cell suicide by dismantling critical cell components.

The autophagic process can be narrowed down to three steps: (1) Membrane isolation at the Phagophore Assembly Site (PAS), from either ER, mitochondria or plasma membrane origin (2) Autophagosome formation, with closure of the macroautophagy vesicle forming a double lipidic bilayer around the organellar/proteic cargo (3) Fusion of the autophagy vesicle with lysosomes, triggering cargo turnover through lysosomal hydrolase-mediated degradation (Das et al., 2012) (See **Figure 8**). At first, yeast genetic screens have allowed the identification of the main genes regulating autophagy. Currently over than 30 genes, named ATG genes standing for AuTophagy-related Genes, are involved in autophagy regulation in yeast, many of these conserved in mammals (Weidberg et al., 2011).

The recruitment of proteins at the PAS require phosphorylation of membrane lipid phosphatidylinositolbiphosphate (PIP₂) into phosphatidylinositol-triphosphate (PIP₃).

Indeed, PIPs are known to participate in the mechanism directing proteins to specific intracellular membrane sites, hence PIP₃ contributes to the autophagic machinery by recruiting several key proteins at the autophagosome formation site (Skwarek et Boulianne, 2009; Burman and Ktistakis, 2010; Weidberg et al., 2011). The enzyme VPS34, from the phosphatidylinositol-3-kinase (PI3K) class III is responsible for the PIP₂ to PIP₃ conversion at the PAS site (Stack et al., 1993).



Figure 8- Autophagosome Formation

Figure 8 – Autophagosome Formation

(a) Recruitment of VPS34 at the initiation site by Ulk1/2 kinase precludes autophagosome formation. The VPS34 complex includes essential regulatory partner VPS15 (not depicted here) and VPS30/Beclin-1, recruited through a scaffold protein which could be either ATG14L (depicted here), UVRAG or RUBICON (not depicted here). (b) Catalytic activity of VPS34 enzyme within the complex allows conversion of PIP₂ into PIP₃ (c) Recruitment of PIP₃-binding protein WIPI1/2 and DFPC1 at the autophagosome initiation site. (d) WIPI2 tethers a protein complex formed by Atg5, Atg12 and Atg16L. (e) Atg5-Atg12/Atg16L complex allows the conjugation of Atg8/LC3 with phosphotidylethanolamine, enabling its membrane insertion, at the autophagosome initiation site. (f) LC3 enables autophagosome elongation and promotes its closure around the cargo targeted to degradation, forming a mature autophagosome (g) The mature autophagosome is fused with acid hydrolase-containing lysosomes. (h) Cargo hydrolysis occurs within the autophagosome fused with lysosomes. (i) Upon cargo breakdown completion, the content of the autophagosome (e.g. amino acids) is released in the cytosol, readily available for biosynthesis. Bcl-2 proteins have been shown to regulate early events of autophagy through the direct binding and inhibition of Beclin-1, impairing VPS34 initiating complex formation. The pro-survival proteins Bcl-2, Bcl-xL and Nrh have been reported to directly bind with Beclin-1 BH3 domain, via their hydrophobic groove.

Three distinct VPS34 protein complexes sharing both VPS15 and VPS30, also called Atg6 or Beclin-1, as required regulatory sub-units, are the main components initiating the autophagosome assembly (Kihara et al., 2001; Funderburk et al., 2010). These three complexes seem to differ in the protein scaffold between VPS34 and Beclin-1, which could be either Atg14L or UVRAG, while a third complex containing both UVRAG and another protein called RUBICON has also been reported.

VPS34-mediated generation of PIP₃ from PIP₂ enables the recruitment of PIP₃ – binding proteins such as Double FYVE-Containing Protein 1 (DFCP1) and WD-40 repeats Interacting with Phosphatidyl Inositol (WIPI) 1/2 at the autophagosome site (Cheung et al., 2001; Axe et al., 2008; Polson et al., 2010). The latter WIPI2 protein, has been shown to tether a complex formed by Atg5, Atg12 and Atg16 at the autophagosome formation site (Romanov et al., 2012; Dooley et al., 2014).

The autophagosome machinery includes two specific ubiquitin-like conjugating system in order to work. Indeed, Atg12 and Atg8, the latter also called LC3, are two ubiquitin homologs. Analogous to ubiquitination, covalent conjugation of Atg12 to Atg5 requires the E1-like enzyme Atg7 and E2-like enzyme Atg10. Similarly, conjugation of Atg8 to the phosphatidylethanolamine (PE) lipid requires E1like Atg7 and E2-like Atg3 protein (Geng et Klionsky, 2008).

Unlike Atg12, pro-Atg8/LC3 is first processed by Atg4-mediated cleavage, exposing a glycin residue at the C-terminus of truncated Atg8/LC3 called LC3-I, on which the PE is conjugated, taking the name LC3-II (Kabeya et al., 2000; Kirisako et al., 2000). Moreover, Atg4 has been shown to cleave again PE-conjugated LC3-II into LC3-I, regenerating the unconjugated pool of LC3 and tightly controlling autophagosome formation (Satoo et al., 2009). LC3 processing from LC3-I to LC3-II is considered a hallmark of autophagy and can be monitored, for example through western-blotting (Klionsky et al., 2016).

The conjugation of Atg12 to Atg5 enables the formation of a stable Atg5-Atg12 complex with the binding partner Atg16. This complex then acts as a scaffolding platform to recruit Atg7 and Atg3-bound LC3, followed by LC3 processing into PE-conjugated LC3-II (Fujita et al., 2008). Furthermore, Atg8/LC3 conjugation to PE has been shown to anchor LC3 in cellular membranes and promote membrane tethering and hemifusion, allowing the elongation until autophagosome vesicle full closure (Nakatogawa et al., 2007).

Finally, the mature autophagosome vesicle is fused with lysosomal content to promote protein degradation. Although this process is still not fully understood, evidences has been found that interaction between Rab7 and RUBICON might recruit Rab7 to the autophagosome membrane (Sun et al., 2010). Rab7 small GTPase is a known regulator of both autophagosome and endosome maturation pathway (Hyttinen et al., 2013). With the help of SNARE proteins, Rab7 has been shown to promote communication, and ultimately fusion between vesicles in the context autophagy, leading to the degradation of the autophagosome content (Nair et al., 2011; Ao et al., 2014).

1.4.2.2. Regulation of Autophagy

In essence, autophagy allows the regulation of cellular amino acids level, especially in scarce conditions. As a consequence, cell signaling pathways must integrate amino acids biodisponibility, as well as sensing energy level available for biosynthesis, represented by Adenosine Tri-Phosphate (ATP). Depending on the received information, the cell must choose between using current resources or undergoing autophagy. In both yeast and human cells, the main sensor probing for these features is the Target Of Rapamycin (TOR) pathway, often called mTOR for mammalian TOR (Feng et al., 2015) (See **Figure 9**).

1.4.2.3. *mTOR* is the pathway regulating Autophagy

The mTOR pathway found its origin and name after a natural product in a soil sample from Easter Island (Rapa Nui) produced by the bacteria *Streptomyces Hygroscopicus*, which was called Rapamycin (Vézina et al., 1975). Rapamycin has shown since impressive anti-fungal, immunosuppressive and anti-tumoral properties (Benjamin et al., 2011). These effects are mediated by the inhibition of the mTOR pathway, more precisely of a protein complex named mTOR Complex 1 or mTORC1. Moreover, mTOR has been shown as a negative regulator of autophagy, hence inhibiting mTOR/mTORC1 with Rapamycin actually triggers a direct autophagic response (Jung et al., 2010). The main sensor protein of the mTOR pathway is called Adenosine Monophosphate-activated Protein Kinase (AMPK), which is inactive at steady state in the cell. As the cell is burning ATP to ADP and AMP, fueling its metabolism and biosynthesis pathways, AMP level can rise to a critical point upon carbon source (glucose) privation. The AMPK protein is sensing cellular AMP level, and will be activated upon reaching a threshold of AMP concentration.

Under normal nutrient conditions, AMPK activity is suppressed and mTORC1 is activated by the RHEB GTPase. Activated mTORC1 then proceeds to inactivate ULK1/2 through inhibitory phosphorylation at the S757 site (Kim et al., 2011). ULK1/2 kinase is essential for autophagy induction, activating both Beclin-1 and VPS34 lipid kinase at the autophagosome assembly site (Russel et al., 2013). When glucose level is lowered, an increase in AMP level results in AMPK activation, which triggers phosphorylation-mediated activation of TSC2. Because TSC1/2 protein is a negative regulator of RHEB, AMPK indirectly inactivates the mTORC1 complex. Therefore, AMPK activation unleashes the mTOR brakes, driving the engagement toward autophagy. To enforce its inhibition over mTORC1, AMPK is also known to phosphorylate Regulatory-Associated Partner of mTOR (RAPTOR) at two conserved sites, enabling its inhibition by the direct binding of the 14-3-3 protein (Gwinn et al., 2008). Finally, AMPK is able to directly activate ULK1/2 by phosphorylating S317 and S777 (Kim et al., 2011).



Figure 9 - mTOR pathway regulates autophagy

Figure 9 – mTOR pathway regulates autophagy

(a) Under normal nutrient availability, glucose represses AMPK activation. RHEB GTPase activate mTORC1 complex, leading to the inactivating phosphorylation of Ulk1/2 at S757 site. With Ulk1/2 inactivation, mTORC1 efficiently suppresses autophagy. Bcl-2 itself can also repress autophagy upstream AMPK, by inhibiting IP3Rs receptor-mediated calcium release and calcium-dependent activation of CAMKIIβ. (b) Upon glucose starvation, AMPK is activated, and can activate TSC1/2 by phosphorylation, which represses RHEB-mediated mTORC1 activation. AMPK inhibits through phosphorylation mTORC1 component RAPTOR, and directly activates by phosphorylation Ulk1/2 at S317 and S777, enabling autophagy. Bcl-2 inhibition is linked with enhanced IP3Rs-mediated calcium release, which leads to CAMKIIβ and subsequent AMPK activation.

1.4.2.4. Crosstalk with the Bcl-2 proteins

Bcl-2 proteins have been studied extensively in the circumstances of apoptosis. On the account of Bcl-2 proteins deep involvement in apoptosis, observation of Bcl-2 control over other types of cellular program can be challenging and stretches the limits of many biological systems. Nevertheless, Bcl-2 proteins have been shown to participate actively in the regulation of autophagy, either by direct protein-protein interaction with autophagic machinery components, or indirect regulation through calcium signaling.

1.4.2.4.1.1. Direct regulation of Autophagy

The first evidence of Bcl-2 proteins participation in autophagy was brought by the observation of a direct interaction between Bcl-2 itself and the autophagy regulator Beclin-1 (See **Figure 8**). Beclin-1 binding to Bcl-2, through a putative BH3 domain contained by Beclin-1, was reported to inhibit VPS34/Beclin-1 complex formation and autophagy initiation (Pattingre et al., 2005). This Bcl-2/Beclin-1 complex can further be regulated by C-Jun N-terminal Kinase JNK1, which can promote dissociation of Beclin-1 from Bcl-2 upon phosphorylating Ser69, Thr70, and Ser87 from Bcl-2 inter-BH4/BH3 domain unstructured loop (Wei et al., 2008).

Soon after, Bcl-xL, Bcl-W and Mcl-1 were shown to interact with Beclin-1, and Beclin-1 BH3 domain was firmly recognized with the resolution of Bcl-xL/Beclin-1 complex crystal structure (Erlich et al., 2007; Maiuri et al., 2007a; Oberstein et al., 2007; Sharma et al., 2013).

On the other hand, Death-Associated Protein Kinase 1 (DAPK1) is able to phosphorylate Beclin-1 BH3 and dissociate both Bcl-2 and Bcl-xL interaction with Beclin-1, allowing engagement into autophagy (Zalckvar et al., 2009a; Zalckvar et al., 2009b). Dissociation of Bcl-2/Beclin-1 interaction can also occur after the Tumor Necrosis Factor Receptor (TNFR)-Associated Factor 6 (TRAF6)-mediated ubiquitination of Beclin-1 (Shi et Kehrl, 2010). Interestingly, Beclin-1 was detected in both mitochondrial and ER fractions, but only ER-targeted Bcl-2/Bcl-xL seemed to negatively regulate autophagic processes (Pattingre et al., 2005; Maiuri et al., 2007b).

However, conflicting reports have arisen on the interaction domains between Beclin-1 and Bcl-2 proteins. A canonical Bcl-2/BH3-only interaction was first described, involving Bcl-2 hydrophobic groove and Beclin-1 BH3 helix, and coherent observations such as titration of the interaction by BH3-mimetic molecule ABT-737 have been made (Oberstein et al., 2007; Maiuri et al., 2007b).

Even though associated with Beclin-1, Bcl-2 have been reported to retain the ability to bind Bax/Bak and its full capacity to regulate apoptosis (Erlich et al., 2007; Ciechomska et al., 2009a). Thereby, in addition to its inability to activate apoptosis, Beclin-1 binding to Bcl-2 might denote leaving the hydrophobic groove available for Bax/Bak BH3 domain fixation, challenging the theory of Beclin-1 BH3-mediated interaction with Bcl-2 proteins (Boya et Kroemer, 2009; Ciechomska et al., 2009b).

Recently, a protein regulating the Bcl-2/Beclin-1 interaction has been found, called NAF-1 standing for Nutrient-deprivation autophagy factor-1 (Chang et al., 2010). NAF-1 is an ER-membrane protein associated with IP3Rs, able to interact with both BH4 and BH3 of Bcl-2 and dissociate the Bcl-2/Beclin-1 interaction (Tamir et al., 2014). Finally, the anti-apoptotic Bcl-2 homolog Bcl-B/BCL2L10/Nrh has been shown to interact with Beclin-1 BH3 domain, and regulate autophagy (Robert et al., 2012).

1.4.2.4.1.2. Indirect regulation of Autophagy

As we have previously discussed, Bcl-2 proteins can control calcium homeostasis at the mitochondria, but also at the ER. From the ER, anti-apoptotic Bcl-2 proteins such as Bcl-2 or Bcl-xL can negatively regulate calcium trafficking through interaction with the IP3 receptors. Bcl-2 proteins can prevent an excessive calcium signaling, that can result into a cytosolic calcium elevation. At some point, the cytosolic calcium concentration can trigger calcium-mediated activation of the CAIModuline-dependent Kinase Kinase-beta (CAMKII- β). Then the CAMKII- β protein can activate AMPK and proceed through the autophagy cascade (Høyer-Hansen et al., 2007) (See **Figure 9**). Consistent observations have also been made, showing the implication of exogenous calcium to induce autophagy (Gao et al., 2008). Calcium has also been reported to trigger autophagy in an AMPK-independent manner, through the activation of CAMKI and monitored by WIPI1-positive autophagosome formation (Grotemeier et al., 2010; Pfisterer et al., 2011).

1.4.2.5. Autophagy – is there a way to cell death?

Given the previous discussion, we have seen that "autophagic cell death" remains more of a morphologic feature rather than a mechanistic way for the cell to die. During *drosophila* development, salivary glands cell death has been shown to be clearly autophagy-related, whereas evidence of a similar autophagic cell death in human has yet to be established (Baehrecke et al., 2003; Kroemer et Levine, 2008).

Still, beclin-1 knock-out mice exhibiting increased tumor formation has underlined the importance of autophagy in oncosuppression at early stages of tumor development in cancer. However, at later stages of the disease autophagy may contribute to tumor development by enhancing tumor mass growth and reducing treatment-induced cell death, through its beneficial effects (Galluzi et al., 2015).

So at the end of the day, is autophagy good or bad for the cell? The answer might be completely context dependent at the cell level (Thorburn A et al., 2014). Interplay between apoptosis and autophagy is known, as for example caspase can cleave Beclin-1 to inactivate its properties to initiate autophagy, further enhancing apoptosis (Wirawan et al., 2010). Altogether, at least half of the human ATG protein can be cleaved by either caspases, or calpains (Norman et al., 2010).

In a recent publication, it was shown that autophagy-mediated degradation of a tyrosine phosphatase called FAP1 sensitizes the cell to Fas ligand-induced apoptosis (Sato et al., 1995; Gump et al., 2014).

Inversely, autophagy can delay MOMP by downregulating the BH3-only PUMA at the mRNA level, allowing cell to survive TRAIL-induced apoptosis (Thorburn J et al., 2014).

To conclude, whether it is good or bad, depending on the cellular or pathological context, autophagy is closely tied with apoptosis, and can make a difference in the commitment into death or survival.

1.5. Physiological roles of Bcl-2 proteins

Bcl-2 proteins have broad roles to play in the homeostasis of many tissues, controlling the self-renewal of cell populations, developmental processes or the maturation of cells from the immune system. We will briefly review the physiological role of Bcl-2 proteins, mainly learnt from reverse genetic experiments in mice (See **Table 2**).

1.5.1. Anti-apoptotic Bcl-2 proteins

Bcl-2 deficient mice can complete embryonic development, but are smaller and display early postnatal mortality associated with polycystic kidney disease and fulminant lymphoid apoptosis. Bcl-2 seems to be critical for kidney development, survival of B and T lymphocytes, as well as melanocyte pigmentation (Veis et al., 1993; Nakayama et al., 1993, 1994; Kamada et al., 1995). Amazingly, most of the defect caused by the loss of Bcl-2 can be counteracted by the additional loss of Bim, confirming the importance of the pro- and anti-apoptotic balance to trim the apoptotic priming of cell population within tissues (Bouillet et al., 2001).

Bcl-xL deficient mice are characterized by a developmental arrest around the thirteenth day of gestation, whereas Mcl-1 deficient embryos failed implantation *in utero* (Motoyama et al., 1995; Rickenberger et al., 2000). Bcl-xL null mice revealed a massive death of immature hematopoietic cells and neurons (Motoyama et al., 1995; Wagner et al., 2000). Studies have shown that concomitant loss of Bax can rescue the neurons abnormal death, but not the hematopoietic cells defect which can only be rescued by the Bcl-xL/Bim double knock-out (Schindler et al., 1997; Akhtar et al., 2008). In addition, Bcl-xL seems to play an important role in the regulation of apoptosis in the postnatal central nervous system (Parsadanian et al., 1998). Conditional knock-out of Mcl-1 was performed to investigate its physiological functions, bypassing the early embryonic mortality phenotype. These conditional knock-out studies showed the central role of Mcl-1 in the survival of B and T lymphocyte along their maturation process (Opferman et al., 2003). Furthermore, conditional Mcl-1 knock-out mice displayed a bone marrow ablation, demonstrating a unique ability of Mcl-1 as a hematopoietic stem cell survival factor (Opferman et al., 2005). Deletion of Mcl-1 in heart was also linked with lethal cardiac failure in mice, and possibly due to Mcl-1 involvement in mitochondrial physiology (Perciavalle et al., 2012; Wang et al., 2013).

Bcl-2 Familly Member	Defects caused by its deletion

Т

Pro-survival members

Bcl-2	Post-natal death through polycystic kidney disease. Abnormal death of B and T Lymphocyte, as well as pigmentation defects.	
Bcl-xL	Embryonic lethal at day 13 in mice. Massive death of immature neurons and haematopeitic progenitors reported.	
Bcl-W	No apparent defects except for male sterility due to defects in spermatogenesis.	
McI-1	Lethal: embryo failed implantation in utero. Conditional KO in adults leads to bone marrow ablation and lethal cardiac failure.	
BfI-1/BcI-2A1	No apparent defects apart from abnormal mast cells and neutrophils apoptosis.	
BCL2L10/Nrh	No phenotype observed in mice. Embryonic lethal in zebrafish.	
Pro-apoptotic multidomains		

Bax	Mild lymphoid hyperplasia and male sterility due to defects in sperm-cells differenciation.
Bak	No obvious defects detected.
Bok	No obvious defects detected.

BH3-only proteins		
Bim	Lymphoid and myeloid hyperplasia causing a fatal autoimun disease. Lack of apoptosis in many cell types.	
Bik	No major defects detected.	
Hrk	No major defects detected.	
Bad	No major defects detected.	
Bid	Fas-ligand induced apoptosis defects in hepatocytes, causing a fatal hepatitis.	
Bmf	No major defects detected.	
Puma	Recapitulates p53 depletion. Profound resistance to DNA-damage induced apoptosis.	
Noxa	Recapitulates p53 depletion. Profound resistance to DNA-damage induced apoptosis.	



Phenotype severity

Table 2 - Bcl-2 proteins knock-out phenotypes

Bcl-W deficient mice do not display a phenotype, apart from the sterility of male offspring due to defect in spermatogenesis (Print et al., 1998; Ross et al., 1998; Russel et al., 2001). Bcl-W has been show to antagonize Bax action and promote survival in Sertoli cells (Ross et al., 2001).

Bcl-2A1/Bfl-1 loss do not lead to any significant phenotype, except from an increase in neutrophil and mast cells apoptosis, as it seems that Bcl-2A1 is an important factor regulating the survival of these cells (Hamasaki et al., 1998; Xiang et al., 2001). A recent study has shown that Bcl-2A1 is a critical survival factor during B cell maturation (Sochalska et al., 2016).

Finally, Bcl2L10/BCL2L10/Nrh knock-out has not showed any observable phenotype in mice, as the offspring is fertile and healthy (Russel et al., 2002). However, invalidation of BCL2L10 zebrafish ortholog Nrz leads to an embryonic lethal phenotype due to a gastrulation defect which is independent of apoptosis (Arnaud et al., 2006; Popgeorgiev et al., 2011).

1.5.2. Pro-apoptotic Bcl-2 proteins

Pro-apoptotic Bcl-2 proteins are more numerous than the anti-apoptotic proteins, and have shown a high degree of redundancy in both their interaction capabilities and physiological functions. As a matter of fact, Bax invalidation displays a partial phenotype in mice, with a mild lymphoid hyperplasia and male sterility whereas Bak invalidation do not produce any sizable defects (Knudson et al., 1995; White et al., 1998; Lindsten et al., 2000). In contrast, double Bax/Bak knock-out leads to a more severe phenotype with developmental and postnatal defects such as interdigital webs, imperforated vaginal canal, an accumulation of neurons and hematopoietic cells, with only 10% of the offspring surviving to adulthood. These observations have shown how much Bax and Bak function overlap to regulate apoptosis in a physiological context, with the ability of a protein to compensate for the loss of another. Loss of Bok is not linked with any discernable phenotype in mice, even with combined knock-out with either Bax or Bak (Ke et al., 2012, 2013).

Among the BH3-only, Bim invalidation seems to induce the more severe phenotype, leading to accumulation of lymphoid and myeloid cells, and increase survival of granulocytes. Defects in T lymphocyte maturation resulting from Bim invalidation can trigger auto-immune diseases from lack of apoptosis (Bouillet et al., 1999, 2002; Villunger et al., 2003a). The knock-out of Puma and Noxa in mice recapitulate the apoptotic deficiencies of p53 loss, for instance cells are more resistant to DNA damage (Jeffers et al., 2003; Shibue et al., 2003; Villunger et al., 2003b). Depletion of Bid is linked with Fas ligand-induced apoptosis in hepatocytes deficiency, causing a fatal hepatitis (Yin et al., 1999). Finally, loss of either Bad, Bik, Hrk or Bmf has no major effects in mice, suggesting they might have an important level of redundancy (Strasser, 2005).

1.6. Receiving orders? Bcl-2 protein functions under cellular signaling tight control

We have seen that the Bcl-2 proteins are regulators of cell life and death. To that extent, Bcl-2 proteins themselves have to be tightly regulated. From the 1.2 part, we can remember that Bcl-2 itself was introduced because aberrant transcription of the *bcl2* gene was observed as a progression marker in B-cell lymphoma (Tsujimoto et al., 1985). Indeed, abnormal expression of different Bcl-2 family member is often found in cancer, and admitted to link tumoral cells with the ability to escape from death (Yip et Reed, 2008). Bcl-2 proteins expression can be modulated by transcription factors, such as NF-kB or p53 (Wu et al., 2001; Katz et Johnson, 2001).

As a matter of fact, many transcription factors can affect the expression of Bcl-2 homologs, thus modifying the equilibrium between pro- and anti-apoptotics. However, apoptosis short kinetic implies a rapid modulation of Bcl-2-mediated life and death decisions, which could not be explained by a transcriptional response. Therefore, how Bcl-2 proteins activity, is finely and quickly regulated emerge as a tempting question.

In the present part, we will mainly discuss the means to dynamically either keep them in check or trigger apoptotic engagement, using post-translational modifications to affect Bcl-2 proteins activity or cellular stability (See Figure 10&11).

1.6.1. Phosphorylation: the signal flare

1.6.1.1. Phosphorylation of Anti-apoptotics Bcl-2 proteins

Early on, it was discovered that growth factors can trigger a cascade of events leading to the phosphorylation of residues in the loop between Bcl-2 BH4 and BH3 domains (May et al., 1994). However, conflicting reports have arisen on the functional effect brought by these modifications (See **Figure 10**).

Some studies have found that the phosphorylation of Bcl-2, especially on S70 residue, was required for its anti-apoptotic function, and antagonized by a direct interaction and subsequent dephosphorylation by PP2A phosphatase (Ito et al., 1997; Deng et al., 1998; Ruvolo et al., 1999).

The kinase PKCalpha (Protein Kinase C alpha) would be responsible for resistance to cell death by phosphorylating Bcl-2 at the S70 site, although this phosphorylation does not abrogate Bcl-2 interaction with death accelerator Bax (Ito et al., 1997; Ruvolo et al., 1998). Mitogen-Activated Protein Kinases (MAPK) such as Extracellular signal – Regulated Kinase 1/2 (ERK 1/2) have also been shown to activate via phosphorylation Bcl-2 (Horiuchi et al., 1997; Deng et al., 2000). Finally, phosphorylation at sites T56, T74 and S87 has been shown to increase Bcl-2 stability, prevent ubiquitinitation and degradation through the proteasomal pathway (Dimmeler et al., 1999; Breitschopf et al., 2000).



Figure 10 - Post-translational modifications of Bcl-2 pro-survival proteins

Figure 10 – Post-translational modifications of Bcl-2 pro-survival proteins

Bcl-2 pro-survival proteins activity can be regulated at the post-translational level.

Bcl-2 has been reported to be inactivated by Raf-1, ERK1/2, PKA, JNK, mTOR, CDK6, p38, or CDK1-mediated phosphorylation at T69, S70 and S87. Controversially phosphorylation at T56, T76, S70 or S87 by ERK1/2 or PKC α has been reported to activate Bcl-2 and enhance its pro-survival activity. Bcl-2 phosphorylation can be antagonized by PP2 α phosphatase. Bcl-2 has been reported in some studies to be conjugated with ubiquitin, although the ubiquitin ligase involved is still unknown.

Bcl-xL has been reported to be the target of inactivating phosphorylation by Raf-1, JNK1/2 or PLK1/3 at S49 and S62.

Mcl-1 protein level is tightly regulated, and phosphorylation status of some residues within or close to its PEST domain serves as a signal to target or prevent its degradation through the ubiquitin/proteasome pathway. Indeed, CDK1/2, JNK1 or ERK1/2-mediated phosphorylation at S64, T92 or T163 sites are signals to prevent Mcl-1 degradation, whereas GSK-3 phosphorylation at S159 is linked with Mcl-1 degradation. Mcl-1 can be ubiquitinated at K136, K194 and K197. Four ubiquitin ligases have been shown to regulate Mcl-1 stability: Mule/ARF-BP1, SCF^{β-TrCP}, SCF^{Fbw7} and Trim17.

Bcl-W has been reported to be inactivated by Akt-mediated phosphorylation, but the precise site is not known.

Bfl-1/BCL2A1 is known to be ubiquitinated at its C-terminal moiety by an unknown ubiquitin ligase.

BCL2L10/Nrh has been reported to be ubiquitinated at K119, K120 and K128, although the ubiquitin ligase involved is not known.

On the other hand, phosphorylation on Serine residues has been shown to inactivate Bcl-2 and was observed after treatment with microtubules inhibitor Taxol (Haldar et al., 1995). The kinase Raf-1 from MAPK pathway seemed to be involved in Bcl-2 inhibitory phosphorylation (Blagosklonny et al., 1996; 1997). PKA-mediated phosphorylation of Bcl-2 has been shown to decrease its interaction with Bax (Srivastava et al., 1998), and might be mediated by the S70 phosphorylation site (Shitashige et al., 2001). The c-Jun N-terminal Kinase 1 (JNK1) exhibits an ability to inactivate Bcl-2 by phosphorylation at T69, S70 and S87 sites, and Bcl-2 mutation Ser70 to Ala (to prevent phosphorylation), or loop deletion between BH4 and BH3 restore Bcl-2 anti-apoptotic function (Yamamoto et al., 1999; Srivastava et al., 1999).

Moreover, phosphorylation defective Bcl-2 mutant, bearing T69A/S70A/S87A mutations, has an increased ability to bind pro-apoptotic Bcl-2 proteins and harbors an enhanced capability to inhibit calcium discharge from ER store (Bassik et al., 2004). It is interesting to note, as we have discussed in the part 1.4.2.2.2.1, that phosphorylation on the three residues T69/S70/S87 also disrupts the interaction between Bcl-2 and Beclin-1 (Wei et al., 2008). Numerous kinases have been found to negatively regulate Bcl-2 by phosphorylation, such as v-cyclin/CDK6 complex (Ojala et al., 2000), CDK1 (Terrano et al., 2010), mTOR (Calastretti et al., 2001; Asnaghi et al., 2004), ERK (Tamura et al., 2004), or p38 (De Chiara et al., 2006). Therefore, phosphorylation of the three conserved residues T69/S70/S87 within the inter-BH4/BH3 loop has an important inhibitory role on the Bcl-2 proteins. Considering the amount of data on Bcl-2 activating phosphorylations against the data regarding inhibitory phosphorylations, we might lean toward the latter.

In a similar manner to Bcl-2, Bcl-xL can also be phosphorylated and inhibited by Raf-1 upon apoptosis inducers treatment, such as microtubule targeting agents (Poruchynsky et al., 1998). Bcl-xL phosphorylation sites appeared mostly in the same loop as Bcl-2, between BH4 and BH3 domains, such as S49 or S62 (Basu et Haldar, 2003; Upreti et al., 2008).

These phosphorylations disable Bcl-xL ability to bind Bax, and could be mediated either by JNK1, MAPK9/JNK2 or Polo-Like Kinase (PLK) 1/3 (Wang et al., 2011; 2012). Moreover, Bcl-xL S49 phosphorylation seems to appear during S and G2 phase of cell cycle, and may govern G2/M transition, i.e. entry into mitosis (Wang et al., 2012; 2014).

Mcl-1 has a longer N-terminal domain compared to other Bcl-2 family proteins, which contains a Proline (P), Glutamate (E), Serine (S), Threonine (T) rich domain, or PEST domain. This domain, present between residues 104 and 175 within Mcl-1, is associated with short protein half-life and proteasome-mediated degradation. Indeed, Mcl-1 PEST domain phosphorylation is closely tied with its cellular stability (Domina et al., 2004). While ERK pathway-mediated phosphorylation at T92 and T163 seems to enhance Mcl-1 stability and promote cell survival (Ding et al., 2008), Glycogen Synthase Kinase 3 (GSK-3) phosphorylates S159 to promote Mcl-1 degradation by FBW7 (Maurer et al., 2006; Inuzuka et al., 2011). It is interesting to take note of how phosphorylation sites so close to each other can have such a differential impact on Mcl-1 half-life.

Furthermore, phosphorylation mediated by CDK1/2 or JNK1 outside Mcl-1 PEST domain at the S64 site may increase Mcl-1 binding to pro-apoptotics (Kobayashi et al., 2007).

Akt-mediated phosphorylation of Bcl-W has been shown to markedly reduce its interaction with proapoptotic Bcl-2 proteins, although no phosphorylation sites have been clearly identified (Garofalo et al., 2008). Finally, Bfl-1/Bcl-2A1 (Vogler et al., 2012) and BCL2L10/BCL2L10/Nrh phosphorylation events have yet to be discovered.

1.6.1.2. Phosphorylation of Pro-apoptotic Bcl-2 proteins

The pro-apoptotic effector protein Bax can be regulated through phosphorylation of its C-terminus S184 site by ERK or Akt pro-survival pathways (Parikh et al., 2004) (See **Figure 11**). Phosphorylation of Bax at S184, which is located in the putative transmembrane C-terminal domain, hindered its localization to mitochondria, enhanced the interaction with anti-apoptotics and prevented apoptosis (Gardai et al., 2004). Besides Akt and ERK, PKC ζ can also inactivate Bax by phosphorylation at S184, which in turn could be reversed by the action of PP2A, restoring Bax full activity (Xin et Deng, 2006; Xin et al., 2007). On the contrary, activating phosphorylation of Bax upon stress conditions have been reported. JNK and p38 kinase seemed to phosphorylate Bax at T167 while GSK-3 β phosphorylate Bax at S163, in both case the net result being mitochondrial translocation of Bax and increased activity (Linseman et al., 2004; Kim et al., 2006). However, comparative analysis have clearly shown that the most important phosphorylation site might be S184, although others might have a less significant impact (Wang et al., 2010).

Bak phosphorylation is also thought to impair its pro-apoptotic activity. It was demonstrated that dephosphorylation of Bak Y108 occurs on the onset of apoptosis, and is required but not sufficient to promote Bak multimerisation and C-terminal transmembrane α 9 helix exposure (Fox et al., 2010). Futhermore, it was shown that negative charge from phosphorylated residues Y110 and S117, located within Bak hydrophobic groove, were responsible for blocking Bak ability to dimerize and engage apoptosis (Azad et al., 2012; Azad et Storey, 2013). Nonetheless, these findings may have to be reassessed as a recent study failed to detect any phosphorylation of Bak, either at steady-state or upon stress induction (Tran et al., 2013).

Bad is known to be phosphorylated mainly at two sites, which are S112 and S136, favoring its interaction with the 14-3-3 protein over Bcl-xL (Downward, 1999). Bad, bound to 14-3-3, displays no evidence of pro-apoptotic activity, hence phosphorylation at S112 and S136 are inhibiting Bad pro-apoptotic function and enhancing cell survival (Zha et al., 1996). Bad phosphorylation at S112 is mediated by PKA (Harada et al., 1999) and Ribosomal S6 Kinase (RSK) 1, the latter downstream of MAPK pathway (Fang et al., 1999; Shimamura et al., 2000), but also JNK1 and Mitogen- and Stress-activated Kinase 1 (MSK1) (She et al., 2002). Bad phosphorylation on S136 site is mainly due to Akt (Datta et al., 1997) or p70S6 kinase (Harada et al., 2001).



Figure 11 - Post-translational modifications of Bcl-2 pro-apoptotic proteins Similarly to pro-survival proteins, pro-apoptotic Bcl-2 proteins could also be regulated at post-translational-level.

Bax can be inactivated by phosphorylation at S184 by ERK1/2, Akt or PKC ζ . On the other hand, Bax can be activated by p38, GSK-3 β or JNK1/2-mediated phosphorylation at S163 or T167. These phosphorylations might be counteracted by phosphatase PP2 α . Bax stability can be also regulated by the ubiquitin proteasome pathway. It has been reported that Parkin promotes Bax ubiquitination, although the precise site is not known.

Bak has been reported to be inactivated by phosphorylation at Y108 and Y110 by PTPN5 tyrosine-kinase. Bak could also be inactivated by phosphorylation on the serine residue S117, but the kinase is still unknown.

Bok has been reported to be ubiquitinated at an unknown site by GP78.

Bim can be inactivated by ERK1/2-mediated phosphorylation at S55, S65, and S100. ERK1/2-mediated phosphorylation of Bim at S65 and S69 triggers its ubiquitination by TRIM2 and subsequent degradation through the ubiquitin/proteasome pathway. JNK has been reported to activate Bim by phosphorylation at T56 and S65.

Bik activity can be enhanced by Casein Kinase II (CK II) –mediated phosphorylation at T33 and S35, whereas ERK1/2 phosphorylation at T124 triggers Bik ubiquitination at K33 by FATE1/RNF183 and its proteasomal degradation.

Bid can be inactivated by Casein Kinase I/II (CK I/II)-mediated phosphorylation at T58. Bid can be ubiquitinated at its N-terminus and C-terminus, the latter by HDM2 ubiquitin ligase.

Bad can be inactivated by phosphorylation at S112, S136, S155 or T201 by either JNK1/2, p70S6K, RSK1, MSK1, PKA, or Akt. Besides, Bad activity can be enhanced by JNK1/2 or CDK2-mediated phosphorylation at T128. Bad phosphorylation can be antagonized by PP1, PP2 α , or Calcineurin phosphatases.

Bmf has been reported to be inactivated by ERK2-mediated phosphorylation at S75 and S77.

Puma activity can be inhibited by a phosphorylation at S10, mediated by a kinase complex comprising IKK1 (IkB Kinase 1), IKK2 and Nemo proteins. Furthermore, Puma has been reported to be ubiquitinated, although both site and ubiquitin ligase are not known.

Noxa phosphorylation at S13 by CDK5 has been reported to impair its pro-apoptotic activity.

Finally, Hrk has not been reported to be regulated by post-translational modification yet.

Bad can be the target of staged phosphorylations, as Bad phosphorylated at S136 promotes phosphorylation at S155 by either PKA or RSK1 (Datta et al., 2000; Tan et al., 2000). This additional phosphorylation further inactivates Bad by lowering its affinity for Bcl-xL, locking any pro-apoptotic activity of Bad (Zhou et al., 2000). Outside the three main residues S112, S136 and S155, Bad can be also phosphorylated at T201 by JNK, inhibiting its binding to Bcl-xL (Yu et al., 2004). Nevertheless, some studies have found that Bad can be dissociated from 14-3-3 through phosphorylation by JNK or cdc2/CDK1 kinase at S128, thereby linking phosphorylation to increased pro-apoptotic activity of Bad (Konishi et al., 2002; Donovan et al., 2002)

Interestingly, Bad phosphorylation on S112 can be reversed specifically by PP2A phosphatase, and this event seems to promote S136 dephosphorylation (Chiang et al., 2003). Calcium-dependent phosphatase Calcineurin has been shown to promote Bad dephosphorylation (Wang et al., 1999). Bcl-2, in addition to Bcl-xL and Bcl-W can also act as a scaffold to target PP1α to Bad, leading to its dephosphorylation (Ayllón et al., 2000; 2001; 2002).

Other BH3-only proteins can be regulated by phosphorylation, such as Bim, on which phosphorylation of S65 or S69 residues by ERK1/2 (Luciano et al., 2003; Ley et al., 2003; 2004). Bim phosphorylation at S55, S65 and S100 can also lower its binding affinity toward Bax (Harada et al., 2004), whereas in another study Bim phosphorylation at S65 lowers its affinity for both Bcl-xL and Mcl-1 (Ewings et al., 2007). Unlike ERK which clearly acts in a pro-survival fashion by promoting Bim degradation through phosphorylation, JNK can also phosphorylate Bim at S65, but promotes apoptosis instead (Putcha et al., 2003). JNK-mediated phosphorylation of Bim at T56 site, located within a dynein binding motif, can lead to Bim release from its anchoring in the cytoskeleton, triggering apoptosis (Lei and Davis, 2003). Phosphorylation of Bid on T58, mediated by Casein Kinase I and II, can prevent its cleavage by caspase-8 following Fas ligand death receptors activation (Desagher et al., 2001).

Bik can be phosphorylate on T33 and S35 residues by Casein Kinase II (CKII), enhancing its proapopotic effects (Verma et al., 2001), although ERK1/2-mediated phosphorylation at T124 promotes Bik degradation through the proteasomal pathway (Lopez et al., 2012).

Puma stability is controlled in a similar manner by phosphorylation at S10 (Fricker et al., 2010). The kinase complex IkB Kinase (IKK) IKK1/IKK2/Nemo would be responsible for S10 Puma phosphorylation (Sandow et al., 2012).

Noxa is phosphorylated by CDK5 kinase at S13 in presence of glucose, promoting cytosolic sequestration and suppressing its pro-apoptotic abilities (Lowman et al., 2010). Amazingly, Noxa S13A mutant sensitizes cells to apoptosis upon glucose starvation. Finally, Bmf phosphorylation by ERK2 at S75 and S77 sites has been shown to reduce its pro-apoptotic functions, although it might involve an unknown mechanism (Shao et Aplin, 2012).

1.6.2. Ubiquitination: the chain of command

As stated above, Bcl-2 proteins can be the target of phosphorylation to regulate both their function and their stability. The latter involves degradation of the protein through the ubiquitin/proteasome degradation system.

Typically, phosphorylation or dephosphorylation of key residues is often the signaling event leading to the targeted protein conjugation with ubiquitin. The ubiquitin conjugation on lysine residues is a multistep process, and requires sequential action of E1 (ubiquitin activating-enzyme), E2 (ubiquitin conjugating-enzyme) and E3 (ubiquitin protein ligase) enzymes. The last E3 enzyme is responsible for the substrate specificity of the conjugation process. Ubiquitin can be grafted as a single molecule (mono-ubiquitination) or as a chain (poly-ubiquitination) on any previously conjugated ubiquitin molecule. Depending on its ubiquitination pattern, the resulting effects range from substrate protein function/subcellular localization alterations to proteasomal-mediated degradation (Mojsa et al., 2014). In this part we will shortly review the Bcl-2 proteins of which expression levels can be regulated by the the ubiquitin/proteasome degradation system (See Figure 10&11).

1.6.2.1. Degradation of Anti-apoptotic Bcl-2 proteins

There is few evidence of active regulation systems for Bcl-2, Bcl-xL, or Bcl-W at the protein level by ubiquitin conjugation. However, phosphorylation of residues T56, T74 and S87 within Bcl-2 inter-BH4/BH3 loop has been shown to prevent degradation (Dimmeler et al., 1999; Breitschopf et al., 2000). Furthermore, it has been shown that Bcl-2 undergoes S-nitrosylation through endogenous Nitric Oxyde (NO) at C158 and C227, which are stabilizing the protein and preventing proteasomal degradation (Azad et al., 2006; Tian et al., 2012).

Ubiquitination is mainly responsible for lowering Mcl-1, Bfl-1/Bcl-2A1 and BCL2L10/Nrh protein level (See **Figure 10**). Mcl-1 ubiquitination is regulated by four different E3 ubiquitin ligases, which are Mule/ARF-BP1 (Zhong et al., 2005), SCF^{β-TrCP} (Ding et al., 2007), SCF^{Fbw7} (Inuzuka et al., 2011) and Trim17 (Magiera et al., 2013). Moreover, Mule/ARF-BP1 interaction with Mcl-1 is happening through a BH3 homology domain contained within the Mule E3 ligase, hence BH3-containing protein may interfere with Mcl-1 ubiquitination and subsequent degradation (Zhong et al., 2005; Warr et al., 2005). Mcl-1 ubiquitination can be reversed by the deubiquitinase USP9X, enhancing Mcl-1 stability and promoting tumor cell survival (Schwickart et al., 2010).

Bfl-1/Bcl-2A1 ubiquitination occurs at the C-terminal of the protein, resulting in a constitutive proteasomal turnover of the protein (Kucharczak et al., 2005; Herold et al., 2006). The E3 ligase responsible for Bfl-1 ubiquitination is still unknown. Interestingly, defects in ubiquitin-mediated Bfl-1 degradation seems to predispose to lymphoma (Fan et al., 2010). BCL2L10/Nrh have been shown to be rapidly degraded by ubiquitination following protein synthesis (Beverly et al., 2012). However, Nrh is

able to interact with ubiquitin-like protein Ubiquilin1, which promotes Nrh mono-ubiquitination and enhance its stability by preventing its degradation. Ubiquilin1-mediated mono-ubiquitination also induced a translocation of Nrh from an exclusively mitochondrial to an extra-mitochondrial localization. Futhermore, in another study, it has been demonstrated that Nrh is poly-ubiquitinated and degraded at steady-state, reducing by a fifth its expression level within the cell (van de Kooij et al., 2013). This study identified K128 as the main ubiquitination site, as well as two putative sites K119 and K120. Finally, as for Bfl-1, the E3 ligase of Nrh has yet to be found.

1.6.2.2. Degradation of pro-apoptotic Bcl-2 proteins

Although the fact that Bax proteasomal degradation contributes to tumor progression has been known for some time, the mechanism involved remained elusive (Li et Dou, 2000) (See **Figure 11**). Recently, the Parkin protein was shown to be Bax E3 ubiquitin ligase, promoting its degradation. Interestingly, Parkin can also prevent Bax localization to mitochondria, the direct interaction between Parkin and Bax leading to Bax retention into the cytosol (Johnson et al., 2012; Charan et al., 2014).

It is unknown whether Bak could be degraded endogenously by ubiquitination in human cells. Nonetheless, it has been reported that E6 proteins from β -HPV type 5 are able to promote Bak degradation through the ubiquitin/proteasome system, decreasing the pool of Bak proteins to engage apoptosis and extending survival of infected cells (Underbrink et al., 2008). Viral E6 proteins could possibly act through the recruitment of HERC1 E3 ubiquitin ligase to Bak (Holloway et al., 2015).

Bok has been shown to be constitutively degraded by ubiquitination, unless it is bound to the IP3Rs receptors (Schulman et al., 2016). Bok ability to bind IP3R1, IP3R2 and to a lesser extent IP3R3 in a small region adjacent to IP3Rs coupling domain (1895-1903 in IP3R1) through its BH4 domain seems to prevent its degradation (Schulman et al., 2013). Additionally, Bok might be ubiquitinated by GP78 E3 ligase at the Endoplasmic Reticulum (Llambi et al., 2016).

Bid seems to be the target of different ubiquitination mechanisms. C-terminal ubiquitination of either full-length Bid or tBid is able to promote its degradation, controlling the overall apoptotic response to the death receptors pathway (Breitschopf et al., 2000). However, after Bid cleavage by caspase-8, ubiquitination of Bid N-terminal fragment might be necessary for its elimination to free Bid BH3 domain, ensuring Bid pro-apoptotic activity (Tait et al., 2007). This ubiquitination may be rather unconventional as the ubiquitin appears to be grafted neither on lysines residues nor the N-termini, but might be serines/threonine/cysteine residues instead. The former ubiquitination of Bid at its C-terminus might involve E3 ligase HDM2 (Hodge et al., 2009).

Finally, as we have discussed previously in the 1.6.1.2. part, Bim, Bik, and Puma protein levels are regulated by ubiquitination. The E3 ligase responsible for Bim ubiquitination is TRIM2 (Thompson et al., 2011), whereas Bik might be ubiquitinated at K33 by the FATE1/RNF183 complex (Lopez et al., 2012; Maxfield et al., 2015) and Puma E3 ligase is still unknown.

2. Calcium: an universal messenger under the tight control of Bcl-2 proteins

Besides being involved in mitochondrial apoptosis, as well as autophagy, Bcl-2 proteins might be major regulators of calcium signaling within the cell. To that end, they have been shown to interact with intracellular calcium channels, at the mitochondria but also at the Endoplasmic Reticulum (ER). Therefore, by controlling intracellular calcium fluxes, Bcl-2 would be able to control virtually any physiological process requiring calcium, including apoptosis as discussed in part 1.4.1.3.3. In the following part, we will mainly focus on how the Bcl-2 proteins can alter calcium signaling at these different compartments.

2.1. Bcl-2 proteins at guard on the ER

From early on, observations have been made that Bcl-2 might be able to suppress cytotoxic calcium signals. Indeed, in IL3-dependent hematopoietic cells, IL-3 withdrawal triggers a calcium dependent apoptotic response which can be repressed by Bcl-2 overexpression (Baffy et al., 1993). While ER calcium pool seemed unaffected by Bcl-2 presence in this study, ER calcium release was decreased, suggesting that Bcl-2 might act to lower calcium efflux at the ER instead. The theory emerged that Bcl-2 anti-apoptotic activity could be exerted following two mechanisms: first through binding of pro-apoptotic Bcl-2 proteins, and second by negative regulation of calcium signaling at the ER membrane (Lam et al., 1994; Magnelli et al., 1994).

This theory was soon put to test when some studies, using state-of-the-art organelle targeted calcium probes, showed that Bcl-2 might decrease ER lumen calcium content (Pinton et al., 2000; Foyouzi-Youssefi et al., 2000; Palmer et al., 2004).

These new findings relied on three concepts to explain the decrease of ER luminal free calcium: Bcl-2 could (1) act as a calcium channel via its membrane insertion domain (α 5 and α 6 helixes) (Schlesinger et al., 1997; Minn et al., 1997; Schendel et al., 1997) (2) interact with SERCA Ca2+/ATP-dependent calcium pump responsible for ER calcium refilling or (3) interact with calcium channels at the ER membrane to promote calcium leak. While the concept (1) proved to be false as Bcl-2 proteins were not found to behave as calcium channels (Chami et al., 2004), the last two concepts were demonstrated to be true. Bcl-2 has been found indeed to interact with the SERCA pump and lower its activity of loading calcium into the ER, thus proving concept (2) (Dremina et al., 2004).

Furthermore, it was observed that Bax/Bak double knock-out Mouse Embryonic Fibroblast (MEF) cells have lower resting calcium content than parental cells, which can be restored by SERCA overexpression (Scorrano et al., 2003). Modulation of inositol 1,4,5-triphosphate receptor type 1 (IP3R1) calcium leak at steady-state by Bcl-2 have been observed, but further studies may be needed to fully validate concept (3) (Oakes et al., 2005). Indeed, selective expression of Bak at the endoplasmic reticulum was showed to trigger ER rapid calcium depletion (Nutt et al., 2002; Zhong et al., 2003). Nevertheless, these results suggest that Bax and Bak may antagonize anti-apoptotic Bcl-2 proteins action at the ER, hence amplifying the apoptotic response whenever triggered.

Finally, Bcl-2 has been found to directly interact with IP3 receptors at the ER, and negatively regulate calcium signals without lowering ER calcium content, consistently with the first studies (Chen et al., 2004). To address the different results discussed above, Bcl-2 phosphorylation status might also be taken into consideration. In fact, Bcl-2 harboring alanine mutations on the three major phosphorylation sites at the loop between BH4 and BH3 domain (T69A, S70A, S87A) displayed an enhanced ability to negatively regulate calcium release (Bassik et al., 2004). Thereby, Bcl-2 proteins can act as signal integrators to control both calcium signal and cell death at the ER, through direct binding and regulation of IP3 receptors. To date, only anti-apoptotic proteins Bcl-2, Bcl-xL, Mcl-1 and BCL2L10 have been shown with the ability to bind IP3Rs (Chen et al., 2004; White et al., 2005; Eckenrode et al., 2010; Popgeorgiev et al., 2011), although pro-apoptotic proteins Bax, Bak and Bik might contribute to control calcium signal by antagonize Bcl-2 homologs at the ER (Oakes et al., 2005; Germain et al., 2002). We will explore in the part below structure and functions of the IP3Rs, as well as the means of Bcl-2 proteins to regulate IP3R-mediated calcium signals.

2.1.1. Closing the gates of the IP3Rs Receptors

2.1.1.1. Structure and functions of the IP3 receptors

It was previously known that the hydrolysis of phosphoinositides such as inositol 1,4,5-triphosphate (IP3) was involved in the mobilization of intracellular calcium pool (Streb et al., 1983; Berridge, 1983; Prentki et al., 1984). Nevertheless, the clues indicating the precise mechanism remained to be found. At first it was suggested that IP3 might have a specific receptor at the ER membrane (Spät et al., 1986). Indeed, this receptor was firmly identified by the team of Kastuhiko Mikoshiba and so-called "IP3 Receptor" (IP3R) or P400 (Furuichi et al., 1989). It was demonstrated that this protein acted not only as a receptor for IP3, but also as a calcium channel (Miyawaki et al., 1990). The IP3R protein was renamed IP3R1 when two distinct genes were found to encode highly similar IP3 receptor, namely IP3R2 and IP3R3, sharing respectively 69% and 62% of homology to IP3R1 (Südhof et al., 1991; Blondel et al., 1993). Many cell types usually feature one predominantly expressed IP3R, although in most of them expression of at least two IP3Rs can be detected (Vermussen et al., 2004).

2.1.1.1.1. IP3Rs main structural elements

IP3Rs are very large proteins, encompassing repectively 2749, 2701, 2670 amino acids from IP3R1, IP3R2 and IP3R3 murine sequence (Blondel et al., 1993). Apart from a membrane spanning C-terminal domain anchored in the ER membrane, the remaining of IP3Rs domains are located at the cytosolic side (Michikawa et al., 1994). According to biochemical studies, IP3R monomers can associate through their C-terminal domains into a tetrameric quaternary structure (Mignery et Südhof, 1990; Maeda et al., 1991). Moreover, IP3R monomers can either form homo-tetramer or hetero-tetramer (Joseph et al., 1995b); Monkawa et al., 1995).

From their large size and membrane spanning domain, complete IP3R structures using current X-ray crystallography or NMR technology is deemed impossible, even in monomeric form. Originally, the precise structure of most IP3Rs domains remained largely unknown, with the exception of N-terminal Suppressor Domain (SD) and IP3 Binding Domain (BD) which were small enough to be crystallized (Bosanac et al., 2002; 2005). However, recent advances in cryo-Electron Microscopy have allowed the resolution of an almost complete IP3R1 homo-tetramer structure at 4.7 Å, unveiling for the first time IP3Rs gating mechanism (Fan et al., 2015).

As IP3Rs are huge proteins, we will shortly discuss their structural elements one by one, using the most characterized IP3R1 mouse amino acid primary sequence as a reference (See Figure 12).

2.1.1.1.1.1. IP3Rs IP3-binding domain (226-578)

The N-terminus of IP3R1 (1-578) is the region necessary for IP3 binding. The essential part required for IP3 binding can be further reduced to the 226-578 fragment, which is called IP3 binding core or IP3 Binding Domain (BD) (Yoshikawa et al., 1996). IP3 BD is composed of a β -domain (226-436) with a β -trefoil fold and an α -domain (437-578) containing alpha helixes organized in an armadillo repeats motif (ARM) (Bosanac et al., 2002).

Interestingly, isolated IP3 BD α -domain has a weak interaction with IP3, while β -domain has none. Nevertheless, mixing together separate recombinant IP3 BD α - and β -domains can reconstitute the same level of IP3 binding as full IP3 BD 1-578, suggesting a cooperative mechanism between α - and β domains, required for ligand binding (Yoshikawa et al., 1999a).

Binding of inositol 1,4,5-triphosphate negatively charged phosphate groups is made possible by hydrogen bonds within the IP3 BD. Key residues binding 4-phosphate are mainly from β -domain (R256, T266, T267, G268, R269, K569) while residues binding 5-phosphate are mostly from α -domain (R265, R269, R504, K508, R511, Y567). Only R568 and K569 can bind 1-phosphate group, underlining the marginal role played by the first phosphate group of IP3 for the fixation to its receptor (Wilcox et al., 1997; Sureshan et al., 2009). With the exception of G268, all IP3Rs types share the same key residues for IP3 binding (Iwai et al., 2007).



Figure 12 - IP3 Receptors architecture

Figure 12 – IP3 Receptors architecture

On the top right, top view of the 3D structure of an IP3R1 tetramer obtained by cryo-electron microscopy (PDB: 3JAV), showing arrangement of each monomer (in red, yellow, green or blue) and the central Ca^{2+} channel. On the top right, the side view of IP3R1 tetramer 3D structure show the smaller transmembrane Ca^{2+} channel and the much larger cytosolic domain. On the bottom, the topology of an IP3R1 monomer, featuring the basic topology originally attributed to IP3R1 mouse primary structure (i.e. amino acid sequence), and the corresponding secondary structure-based topology from the 3D structure recently obtained (Fan et al., 2015). <u>Primary structure</u>: Suppressor Domain (SD), IP₃ Binding Domain (IP₃ BD) subunit α and β , Modulatory and Transducing Domain (MTD) I, II and III, Channel-Forming Domain (CFD), and Coupling Domain (SD).

<u>Secondary structure</u>: β-Trefoil fold (β-TRF) domain 1 and 2, Armadillo Repeat Motif (ARM) domain 1, Helical Domain (HD), ARM2, ARM3, Intervening Lateral Domain (ILD), TransMembrane Domain (TMD), LiNKer domain (LNK) and C-terminal domain (CTD). The TMD domain contains six TransMembrane (TM) helixes noted TM1 to TM6. The TM6 helix is acting as the Ca²⁺ channel. In addition to the IP3 binding pocket, there are two conserved putative calcium ion binding sites within IP3 BD (Bosanac et al., 2002). The first is located in the β -domain (E246, E425, D426, E428) whereas the second spans across the α - and β -domains (E283, E285, D444, D448).

2.1.1.1.1.2. IP3Rs Suppressor domain (1-225)

At the very N-terminus of IP3Rs receptor (1-225 region), the so-called "Suppressor Domain" or SD is able to decrease, by its presence, IP3 affinity to IP3Rs (Yoshikawa et al., 1996). Without the SD, all IP3Rs BD displayed an *in vitro* apparent affinity constant (K_d) for IP3 around 2 nM, in contrast with respective K_d of 49 nM, 14 nM and 163 nM for IP3R1, IP3R2 and IP3R3 SD-BD constructions (1-604) (Iwai et al., 2007). Despite these data, IP3Rs SD alone are not sufficient to explain such drastic affinity differences between IP3Rs, as chimeric SD-BD proteins do not have the same affinity as the SD donor receptor type (Iwai et al., 2007). Therefore, the SD-BD pair of each receptor type may have a unique interface determining the affinity for IP3. Shaping this interface, seven common SD key residues were found between the three receptor types L30, L32, D34, R36, R54, K127 (Bosanac et al., 2005).

Besides modulating IP3 affinity, IP3Rs SD is critical to calcium release function. Indeed, deletion of the SD results in a calcium release deficient, but IP3 binding competent receptor (Uchida et al., 2003). According to earlier studies, an interaction between the N-terminus and channel forming domain at the C-terminus of IP3R1 was reported (Joseph et al., 1995a; Yoshikawa et al., 1999a; Boehning et Joseph, 2000). This interaction is mediated through a hydrophobic residue within the suppressor domain, Y167 for IP3R1 or W168 for IP3R2 and R3 (Yamazaki et al., 2010; Chan et al., 2010). Furthermore, mutation of these residues into alanine abolishes IP3Rs opening and calcium release, but without impairing IP3 binding (Yamazaki et al., 2010). Inversely, mutation of residues L30, L32, D34, R36, R54, K127 involved in modulating IP3 affinity had no effect on calcium release (Bosanac et al., 2005; Yamazaki et al., 2010). To conclude, the interaction of IP3Rs SD with the IP3Rs C-terminus is essential to promote IP3Rs opening and calcium release from the ER.

2.1.1.1.1.3. IP3Rs Modulatory domain (579-2275)

The central Modulatory and Transducing Domain (MTD) of IP3Rs connects the IP3-binding region (IP3 BD and SD) with the channel forming domain. Recent insights into IP3R1 structure by cryo-EM have shown that this domain is composed of an α -helical domain, followed by two armadillo solenoid folds named ARM2 and ARM3 (Fan et al., 2015). Moreover, deletion of fragment 651-1130 results in an IP3 binding competent receptor but impairs calcium channel activity (Uchida et al., 2003).

The modulatory domain is thought to be an important regulatory region, able to bind numerous proteins involved in the modulation of IP3Rs mediated calcium release.

The IP3Rs MTD is connected to the next channel-forming domain by the Intervening Lateral Domain (ILD) from residues 2193-2272 (Fan et al., 2015).

2.1.1.1.1.4. IP3Rs Channel-forming domain (2276-2589)

Critical for the transfer of calcium ions across the ER membrane, the channel forming domain of IP3Rs is composed of six α -helical transmembrane domains, named TM1 to TM6 (Michikawa et al., 1994; Galvan et al., 1999). Expression of the IP3Rs channel domain only has been shown to induce calcium leakage and emptying ER calcium pool (Nakayama et al., 2004). Although the channel domain features structural similarities with other ion channels, implying that selectivity for specific ions may be rather poor (Williams et al., 2001), key residues within the TM6 domain such as V2548 and D2550 seem to enhance selectivity toward calcium (Boehning et al., 2001).

The anchoring of IP3Rs at the ER is mediated by TM1 and TM2 domains (Galvan et al., 1999; Pantazaka et Taylor, 2010). There is still no evidence of the function of the TM3 domain. The loop linking domain TM4 and TM5 is critical to regulate calcium channel opening, as it interacts with the conserved hydrophobic residue Y167 or W168 located in the Suppressor Domain, to coordinate IP3Rs channel opening (Schug et Joseph, 2006; Yamazaki et al., 2010; Chan et al., 2010; Bhanumathy et al., 2012). It is thought that the assembly of IP3Rs into a tetramer is modulated by TM5 and TM6 (Galvan et al., 1999). Finally, IP3R1 3D structure firmly demonstrated that TM6 forms the calcium channel domain, with a loop between TM5 and TM6 within the ER lumen exerting a selective filter activity (Schug et al., 2008; Fan et al., 2015).

2.1.1.1.1.5. IP3Rs Coupling Domain (2590-2749)

At the C-terminal end of the IP3Rs receptor, the Coupling Domain (CD), is located at the cytosolic side of the ER membrane. It is divided into two distinct domains: a helical linker (LNK) from residues region 2601 to 2680 connected to the calcium channel TM6 helix, and the a 80 Å helix at the C-terminal called CTD standing for C-Terminal Domain (Fan et al., 2015). It has been demonstrated that a highly conserved region between residues 2629 and 2654 help the receptor to assemble in a tetramer together with TM5&6 domains (Galvan et Mignery, 2002). Another region of the CD (2694-2721), which is required for channel activity, has been shown to interact with the linker between TM4 and TM5 (Schug et Joseph, 2006; Bhanumathy et al., 2012). IP3Rs CD also interacts with the IP3 BD, connecting the N-terminal and C-terminal end of the receptor (Fan et al., 2015).

It is thought that residues H2630 and H2635 facilitate this contact within the CD, but also affects receptor global affinity for IP3, underlining the interconnectivity and complexity of the IP3Rs (Uchida et al., 2003; Bhanumathy et al., 2012).

Finally, due to its close proximity with both the N-terminal IP3 binding region and the TM4-TM5 linker, the CD is thought to be involved in the propagation of conformational energy generated through the binding of IP3 on IP3Rs (Yamazaki et al., 2010).

2.1.1.1.2. IP3Rs-mediated calcium release

2.1.1.1.2.1. IP3Rs-channel opening

At the onset of IP3Rs calcium signals, generation of IP3 is made through a PhosphoLipase C (PLC) catalyzed hydrolysis of PIP2 (PhosphatidylInositol 4,5-biPhosphate). PIP2 is hydrolyzed into DiAcylGlycerol (DAG) which stays anchored at the plasma membrane, while IP3 is released and freely diffuse into the cytosol. PLC is activated upon fixation of an extracellular ligand, either through G protein-coupled or tyrosine kinase receptors at the plasma membrane (Kadamur et Ross, 2013). For example, addition of histamine in the extracellular medium is a typical external stimulation that will generate IP3 upon histamine receptor-mediated activation of PLC (Bootman et al., 1992). Upon IP3 binding to the IP3 receptor, conformational changes will ultimately trigger the channel opening (See **Figure 13**). (1) Firstly, the α and β domain of the IP3 Binding Domain came closer from each other as IP3 binding reduce by 8° the angle between the two domains. (2) Subsequent rotation of the Suppressor Domain by 9° toward the IP3 BD domain α result in the translation of the conserved W167 residue by 3.7 Å (Seo et al., 2012).

The conformational energy generated by IP3 binding is released following two distinct routes to trigger IP3Rs opening. The energy from (1) is transferred from N-terminal IP3 BD β domain to the close Coupling Domain CTD, which apply pressure on the Coupling Domain LNK region. Through its direct connection to the Pore forming domain, the Coupling Domain LNK allows the open conformation of the calcium channel TM6 helix (Fan et al., 2015). Conformational energy stored from (2) is transferred from the Suppressor Domain to the ARM3 region of the Modulatory and Transducing Domain. As the ARM3 is directly adjacent to the ILD region connected to the Pore forming domain, conformational energy might be transferred to finally promote the open conformation of the calcium channel (Fan et al., 2015).

However, the IP3R1 structure obtained from cryo-EM does not show a direct connection between W167/Y168 residues of the Suppressor Domain and the linker loop between TM4 and TM5 helix, so critical to promote channel opening in previous studies (Yamazaki et al., 2010; Chan et al., 2010; Fan et al., 2015). Further characterization of these interfaces are needed to bring definitive answers about the IP3R channels opening mechanism.

2.1.1.1.2.2. IP3-mediated calcium signals

Calcium signals resulting from IP3Rs opening are often referred to as calcium oscillations, and propagate into the cytosol as regenerative calcium waves. Typically, the opening of an IP3 receptor cluster results in a local (< 6µm radius) elevation of calcium concentration, from 50-100 nM at steady-state to ~ 600 nM for a limited duration (<1 s) (Berridge et al., 2003). Sustained oscillations or further calcium signals propagation require either opening of neighboring channels or modulation by partner proteins. It is known that IP3 receptor can participate in the former through a mechanism called CICR (Calcium-Induced Calcium Release), as they exhibit sensitivity to calcium, which can exert an allosteric regulation and promote IP3Rs opening (Foskett et al., 2007). CICR can be mediated by IP3Rs alone or in combination with another type of calcium channel more sensitive to calcium, the Ryanodine Receptors (RyR), depending on the cell type. Through CICR, calcium signal can propagate from a receptor cluster to another into the whole cytosol. Finally, from ER-located IP3Rs cluster calcium can be transferred to the mitochondria at organelle contact sites called MAM (Mitochondria-Associated Membranes) (Vance, 2014; Prudent et al., 2015).



Figure 13 - IP3 Receptors calcium release mechanism

Figure 13 – IP3 Receptors calcium release mechanism

Schematic representation of IP3R1 domains directly involved in Ca²⁺-release activity, from N-terminal to C-terminal: Suppressor Domain (SD), IP₃ BD α and β subunits, Armadillo Repeat Motif domain 2 (ARM2), ARM3, Intervening Lateral Domain (ILD), TransMembrane (TM) helixes from TM1 to TM6, LiNKer domain (LNK) and C-Terminal Domain (CTD).

(a) At steady-state, the Ca²⁺ channel TM6 helix is closed and IP₃ BD is found in an open state without IP₃ bound. Molecular contacts between SD and ARM3, but also IP₃ BD β subunit and CTD, ensure IP3R1 close conformation.

(b) Upon ligand binding, a two-step conformational rearrangement occurs. First (1) IP₃ binds to IP₃ BD, triggering a conformational movement reducing by 8° the angle between IP₃ BD α and β subunits. In addition, a 9° rotation of the SD toward IP₃ BD α pushes forward by 3.7 Å the conserved residue W167 within IP3R1 SD. In a second time (2) CTD domain is repulsed by IP₃ BD closure on its ligand, transferring conformational energy to the LNK then to the TM6 Ca²⁺ channel, promoting its opening. On the other side, SD pushes away ARM3 domain, which transfers the corresponding conformational energy to the ILD domain, then to the TM helixes, leading to the TM6 Ca²⁺ channel opening and Ca²⁺ release (Fan et al., 2015). Previous studies have shown the involvement of a hydrophobic interaction between SD and the linker separating the TM4 and TM5 helixes at the cytosolic side (Yamazaki et al., 2010; Chan et al., 2010). This interaction has been shown to repulse the linker in the direction of the cytosol, and the induced conformational changes linked with IP3R1 opening. However, the cryo-electron microscopy 3D structure of IP3R1 failed short of showing such an interaction (Fan et al., 2015). Further studies are needed to clarify this point.

2.1.1.2. Regulation of IP3Rs by the Bcl-2 proteins

As we have seen in part 1., Bcl-2 proteins are master regulators of apoptosis at the mitochondria. Besides, mitochondrial calcium appears to be one of the trigger events of MOMP and apoptosis (See part 1.4.1.3.3.). Therefore, it was thought that Bcl-2 proteins could indeed regulate calcium and cell death, upstream at the ER. The interaction between anti-apoptotic Bcl-2 homologs and the IP3 receptors should be the most important way of regulating calcium homeostasis and apoptosis at the ER (See **Figure 14**). Bcl-2 and Bcl-xL have been shown to interact with all three isoforms of IP3Rs (Chen et al., 2004, White et al., 2005).

The interaction of Bcl-2 within the IP3Rs have been shown to take place between residues 1347 and 1426, further narrowed down to a 20 amino acid peptide encompassing residues 1389 to 1408, corresponding to the ARM2 domain within IP3R1 MTD (Rong et al., 2008). Bcl-2 N-terminal BH4 domain is required for its interaction with the IP3Rs, and is sufficient to inhibit IP3Rs-mediated calcium signaling (Rong et al., 2009). Rong and colleagues have shown that it was possible to disrupt the Bcl-2/IP3R1 interaction using the peptide IP3R1 1389-1408 as a decoy for Bcl-2 binding. This peptide has a unique ability to reverse Bcl-2 negative regulation on IP3Rs-generated calcium signals and suppress Bcl-2 cytoprotective capability.

On the other hand, Bcl-xL might preferentially interacts with the C-terminus of IP3Rs. Bcl-xL does not decrease IP3Rs calcium release, but instead promotes calcium oscillations in response to small amounts of IP3 (White et al., 2005). These calcium spikes are transmitted from ER to mitochondria at organelle contact sites, and enhance metabolism efficiency, monitored by NADH production (White et al., 2005). In addition, it has been demonstrated that anti-apoptotic effect of Bcl-xL in resting cells is partly due to the presence of any IP3 receptor isoforms, further demonstrating the importance of Bcl-xL-mediated calcium oscillations in cell survival (Li et al., 2007). Amazingly, a form of the oncogene K-Ras called K-Ras4B associates with Bcl-xL and IP3Rs, disrupts the ability of Bcl-xL to promote pro-survival calcium oscillations and triggers apoptosis (Sung et al., 2013).

In another study Bcl-2, Bcl-xL, but also Mcl-1 were shown to interact with a portion of the C-terminal domain from either IP3R1, IP3R2 or IP3R3, comprising the TM6 calcium channel helix, with LNK and CTD domains (Eckenrode et al., 2010). Although this study provides a narrower interaction site for Bcl-xL, it was quite surprising that Bcl-2 could also interact with IP3Rs C-terminus. This was explained by the discovery of two BH3 conserved motifs in the Coupling Domain of IP3R1. The first one is an α -helix of the LNK domain (H1), the other one contained in the CTD domain, which is the last α -helix of IP3R1 (H4). Furthermore, destabilization of Bcl-xL BH3 binding pocket hampered its interaction with H1 and H4 helix containing BH3 motifs (Yang et al., 2015). Hence, the Coupling Domain of IP3Rs can be the harbor of anti-apopotic Bcl-2 proteins through canonical BH3/hydrophobic groove interaction.



Figure 14 - IP3 Receptors regulation by Bcl-2 proteins

Figure 14 – IP3 Receptors regulation by Bcl-2 proteins

Schematic representation of IP3R1 domains directly involved in Ca^{2+} -release activity, from N-terminal to C-terminal: Suppressor Domain (SD), IP₃ BD α and β subunits, Armadillo Repeat Motif domain 2 (ARM2), ARM3, Intervening Lateral Domain (ILD), TransMembrane (TM) helixes from TM1 to TM6, LiNKer domain (LNK) and C-Terminal Domain (CTD).

Bcl-2 has been shown to interact with its BH4 domain in the ARM2 region (amino acids 1389 to 1408) and inhibit IP3R1-mediated Ca²⁺-release. A peptide derived from IP3R1 interaction site with Bcl-2 called 1389-1408 peptide or BIRD-2 was shown to reverse Bcl-2 inhibition of Ca²⁺-release. Bcl-xL has been reported to bind with its hydrophobic groove two distinct BH3-containing sites, one located in the CTD and the other one in the LNK. Bcl-xL enhances at steady-state pro-survival calcium oscillations, and might gate IP3R1 in stress conditions to prevent excessive Ca²⁺-release. Mcl-1 was shown to bind a region encompassing LNK and CTD and negatively regulate IP3R1-mediated Ca²⁺-release. Mcl-1 interaction domain with IP3R1 has yet to be determined. Nrh BH4 domain was reported to bind IP3R1 and inhibit IP3R1-mediated Ca²⁺-release, although IP3R interacting region is not known. Bok is known to interact with the region 1895-1903 of IP3R1, but no functional effect has been clearly established.
Moreover, the selectivity profile between Bcl-2 and Bcl-xL for either the 1389-1408 ARM2 residues or the Coupling Domain of IP3R1 was determined by Monaco and colleagues. Bcl-2 was found to interact at both sites, whereas Bcl-xL only at the Coupling Domain (Monaco et al., 2012b). This selectivity is explained by the residue K17 in Bcl-2 BH4 domain, which is replaced by an aspartic acid in the corresponding position 11 of Bcl-xL. Swapping the residues in Bcl-xL, through a D11K mutation, confers properties similar to Bcl-2 and allows interaction between Bcl-xL and the peptide 1389-1408 (Monaco et al., 2012a).

Thus Bcl-xL BH4 alone could not bind or regulate IP3Rs, but its BH3 binding pocket is required instead. Bcl-2 might also bind to IP3Rs C-terminal domain via its BH3 binding pocket, even though it preferentially interacts with the 1389-1408 peptide.

It has been reported that Bcl-2 might regulate calcium release from the ER by IP3R1 phosphorylation at the S1755 site, which is known to enhance IP3R1 activity (Oakes et al., 2005). Recently, it has been found that Bcl-2 can be a scaffold to recruit at the ER DARPP-32 kinase and Calcineurine phosphatase. Upon calcium release, calcium binds and activates calcineurine, which dephosphorylates DARPP-32 and breaks DARPP-32 inhibition of PP1. PP1 is then freed to dephosphorylate IP3R1 at S1755, thus reducing IP3-induced calcium release (Chang et al., 2014).

Bok may be the only pro-apoptotic Bcl-2 protein reported to bind IP3Rs. The putative BH4 domain of Bok has been shown to interact with IP3R1 between residues 1895 and 1903 (Schulman et al., 2013). There is no evidence of direct functional effects of Bok binding toward IP3Rs activity, although Bok might protect IP3Rs from caspase-3 mediated proteolytic cleavage during apoptosis (Hirota et al., 1999). It has been proposed that caspase-3 cleaved IP3R1 could still act as an unregulated calcium channel, contributing to final stages of apoptosis through calcium release (Assefa et al., 2004; Nakayama et al., 2004), although the latter is somewhat contested (Akimzhanov et al., 2013).

Last but not least, BCL2L10/Nrh ortholog in zebrafish Nrz has also been reported to interact with the IP3R1 receptor through its BH4 domain. Although this interaction leads to a decrease in IP3-induced calcium release, the IP3R1 domain involved in this interaction has yet to be found.

2.1.2. Locking the RYRs back door

Besides the regulation of IP3 receptors, it is worth to mention that very recently Bcl-2 proteins have been shown to regulate as well another type of ER calcium channels, the Ryanodine Receptors (RyRs). The RyRs receptor family, as for IP3Rs, comprises three distinct members. Ryanodine receptors have been identified to be the main component of Calcium-Induced Calcium Release, as calcium ions can promote their open state to a greater extent than IP3Rs (Lai et al., 1988; Hymel et al., 1988).

Early studies have demonstrated a similar topology between IP3Rs and RyRs, especially in the N-terminus.

The N-terminal RyR region, corresponding to the SD and IP3 BD domain of IP3Rs, displays highly similar structural properties and allosteric activation mechanism (Yuchi and Van Petegem, 2011; Seo et al., 2012). Structural similarities are also found at the C-terminus, with a 6 TM region membrane-spanning calcium channel, while the remaining domains are quite divergent from IP3Rs structure, forming several huge solenoid-related folds (Zalk et al., 2015; Yan et al., 2015). The large size of the RyRs, over 5000 amino acids, allows the binding of multiple regulatory partners. Among them, features Bcl-2 and Bcl-xL but no other Bcl-2 homologs to date.

Bcl-2 has been shown to bind RyRs through its BH4 domain at a conserved site in both IP3Rs and RyRs family, corresponding to the 1389-1408 region of IP3R1 (Vervliet et al., 2014). It has been demonstrated, as for IP3Rs, that Bcl-2 BH4 alone was sufficient to suppress RyRs-mediated calcium signaling. In addition, pharmacological inhibition of Bcl-2 BH3 binding pocket has clearly shown that it had no impact on its RyRs negative regulation capability, firmly uncoupling BH3 binding properties from RyRs modulation (Vervliet et al., 2015b).

Bcl-xL interaction with RyR3 was reported, though the interaction site was not specified and it is not known whether it can interact with the two other RyRs isoforms (Vervliet et al., 2015a). Bcl-xL BH4 domain alone was sufficient to inhibit RyR3-mediated calcium release. Nevertheless, a point mutation in Bcl-xL BH3 domain (K87D) seems to impair its binding and ability to regulate RyR3. The functional impact of this point mutation needs to be clarified, as the K87D mutation may hinder conformational exposure of the BH4 N-terminal moiety, which does not need to happen when the peptide corresponding to the BH4 domain of Bcl-xL is alone in solution.

2.2. Regulation of mitochondrial calcium by Bcl-2 proteins

Mitochondria is an essential part of calcium homeostasis within the cell. The ER represents the most important cell calcium store and controls the upstream part of calcium signals via the IP3Rs, whereas mitochondria is role is to tame calcium oscillations and buffer cytosolic calcium levels.

A constant amount of calcium ions must be present within the mitochondrial matrix to keep the TCA cycle running (Wan et al., 1989). However, as we have seen in part 1.4.3.3.3.1., calcium uptake capacity of the mitochondria is rather finite, eventually causing mitochondrial burst, releasing cytochrome c and triggering apoptosis. Therefore, mitochondrial calcium uptake might be tighly regulated. This calcium uptake is mainly mediated at the OMM by the VDAC channel family, and at the IMM by the Mitochondrial Calcium Uniporter (MCU). Bcl-2 proteins can exert a regulation on mitochondrial calcium uptake, and most of the found evidence has depicted their involvement at the OMM in the control of VDAC channels (See Figure 15).

In this part, we will shortly review the role of the VDAC channel family in calcium transport and the involvement of Bcl-2 protein in the regulation of this activity.

2.2.1. VDAC-mediated calcium transport

The VDAC family, standing for Voltage-Dependent Anion Channel, comprises three highly homologous isoforms of integral membrane proteins about 300 amino acids long. X-Ray crystallography structures have revealed that VDAC1 is a β -barrel protein composed of 19 β -strands. In addition, an N-terminal α -helix of 26 amino acids, horizontally oriented inside the barrel, is responsible for VDAC selectivity toward ions and other metabolites (Bayrhuber et al., 2008; Ujwal et al., 2008).

At its maximal cavity opening, which is 27 x 14 Å, VDAC1 would let pass many metabolites if it was not exhibiting selectivity for some of them. VDAC opening can be controlled by mitochondrial membrane potential, for instance applying a negative or positive tension between 30 to 40 mV amplitude closes the channel within a second (Shoshan-Barmatz et al., 2010).

On the other hand, between -10 and +10 mV, VDAC can stay open for up to 2 hours. Interestingly, negative voltage (-40 mV) gating of VDAC promotes its closure but enhances specificity for calcium as well as increases calcium uptake (Tan et Colombini, 2007).

Moreover, a pharmacological VDAC blocker G3139 was shown to promote VDAC closure, but increases production of mitochondrial metabolism byproducts such as ROS (Reactive Oxygen Species) and sensitizes to calcium-driven apoptosis (Lai et al., 2005; Tikunov et al., 2010).

2.2.1. Regulation of VDAC channels by the Bcl-2 proteins

Bcl-xL was the first protein from the Bcl-2 family reported to functionally interact with VDAC1 via its BH4 domain (Shimizu et al., 1999; 2000a). These early reports showed that VDAC may oligomerize with Bax to form large pores involved in cytochrome c release, which formation could be antagonized by Bcl-2 or Bcl-xL (Shimizu et al., 2000b; 2001).

However, these findings are now largely contested, as VDAC1 and VDAC3 knock-out in cell lines failed to protect from mitochondrial cytochrome c release, further supported by the fact that VDAC2 knock-out even accelerated apoptosis (Cheng et al., 2003; Baines et al., 2007). Accordingly, VDAC1 and VDAC3 KO mice were viable and displayed only a mild phenotype, whereas VDAC2 KO proved to be embryonically lethal (Anflous et al., 2001; Sampson et al., 2001; Baines et al., 2007).

Although VDAC contribution to apoptosis as an essential component directly responsible for cytochrome c release may have been largely overestimated, it remains a major player of mitochondria calcium import. Indeed, it is the closure of VDAC, which enhances calcium flow through the OMM, and not VDAC opening that can trigger apoptosis upstream MOMP, through calcium-mediated mPTP opening (Rostovtseva et al., 2005) (See **Figure 16**).



VDAC1 3D structure top view

β-Barrel structure (19 β-strands)



VDAC1 3D structure side view

Figure 15 - VDAC channels topology

Figure 15 – VDAC channels topology

On the left, VDAC1 3D structure top view (PDB: 3EMN) showing the pore structure, with the N-terminal gating alpha-helix displayed in red. On the right, VDAC1 3D structure side view, showing the β -barrel fold, composed of 19 β -strands.

Bcl-xL and Mcl-1 have been reported to interact with VDAC1 and VDAC3 but not VDAC2, enhancing calcium transfer to mitochondria and metabolic efficiency (Huang et al., 2013; 2015). The functional implications of this interaction is nevertheless still in debate for Bcl-xL. Indeed, Bcl-xL BH4 domain but not of Bcl-2 has been shown to protect from toxic ER to mitochondria calcium transfer, mediating Bcl-xL anti-apoptotic activity (Arbel et al., 2012; Monaco et al., 2015). Amazingly, peptides derived from the N-terminal helical domain of VDAC1 are able to counteract the effect of Bcl-xL and Mcl-1 in the corresponding studies. Thereby, their interaction site may probably be the N-terminus channel gating helical moiety of VDAC channels.

Bcl-2 anti-apoptotic activity could be reduced by VDAC1 N-terminus-derived peptides, suggesting an interaction between VDAC1 and Bcl-2 (Arbel et Shoshan-Barmatz, 2010). However, proof of this interaction has yet to be found, especially with the added elements of the latest studies and would be unlikely (Monaco et al., 2015).

Bcl-xL has been recently reported to interact also with VDAC2, as part of a complex including BECN1 and MYBL2 proteins, which is involved in autophagy suppression during mammalian ovary development (Yuan et al., 2015).

VDAC2 KO mice lethal phenotype can be explained by VDAC2 anti-apoptotic activity to inhibit Bak at the mitochondria (Cheng et al., 2003). VDAC2 is required for tBID induction of apoptosis, as recruitment to the mitochondria of Bak seems VDAC2-dependent and accelerates apoptosis upon induction (Roy et al., 2009).

Furthermore, disruption of the VDAC2/Bak interaction by Bcl-xS has been reported to trigger apoptosis in melanoma cells (Plotz et al., 2012). Bak seems to interact with VDAC2 through its C-terminal transmembrane domain (Lazarou et al., 2010). Unlike Bcl-xL, Bak was reported to interact with a conserved domain specific to VDAC2 within the β -barrel structure, between residues 123-179 (Naghdi et al., 2015).

Finally, despite a lack of evidence it can be tempting to speculate that Bak has the ability to regulate calcium transfer properties of VDAC2. In fact, it was reported that the BH3-only protein Bid, but not Bax has the ability to regulate VDAC channels activity by promoting their closure, accelerating apoptosis (Rostovtseva et al., 2004).



Figure 16 - VDAC regulation by the Bcl-2 proteins

Figure 16 – VDAC regulation by the Bcl-2 proteins

Bcl-2 proteins can regulate VDAC-mediated Ca²⁺-trafficking at the mitochondria.

Bcl-xL BH4 and Mcl-1 can interact with VDAC1 and 3 N-terminal gating helix (displayed in red), enhance Ca²⁺ transfer to mitochondria and metabolic efficiency. Moreover, Bcl-xL BH4 can protect from calcium-driven apoptosis. Mcl-1 interacting domain with VDAC1/3 has yet to be determined.

Bak has been reported to interact with VDAC2 123-179 region (displayed in blue) through its C-terminal domain. This interaction allows Bak sequestration in an inactive form, hence counteract Bak pro-apoptotic activity. However, it is not known whether Bak interaction with VDAC2 can affect Ca^{2+} signals.

Bid has been shown to induce VDAC closure, hence enhance VDAC-mediated Ca²⁺ transfer. However, the respective interacting domains between these two proteins are not known.

3. Pharmacological modulation of Bcl-2 proteins: from investigating their function to treatment of human pathologies

Human pathologies have been the driving force for the discovery of the Bcl-2 protein family. Since their involvement in diseases has been known for more than 30 years, solutions have been proposed to target their activity and abrogate, for instance, tumor cells resistance to chemotherapy. In a first part, we will quickly review the altered state of Bcl-2 proteins in pathologies, and we will focus along a second part on the means currently known to modulate their activities (See **Table 3**).

3.1. Involvement of Bcl-2 proteins in human pathologies

3.1.1. Bcl-2 proteins altered expression in cancer

3.1.1.1. Modulation of Bcl-2 protein levels in cancer

The first mechanism of Bcl-2 overexpression was related to the t(14;18) chromosomal translocation, altering bcl-2 gene structure by trading its own promoter to a stronger immunoglobulin promoter in Bcell lymphoma (Tsujimoto et al., 1985). Besides gene structure modification, copy number variation is also found to enhance overall expression of Bcl-2 in non-Hodgkin's lymphoma as well as in small cell lung cancers (Monni et al., 1997; Olejniczak et al., 2007). Interestingly, copy number variation of the *bcl-2* genomic region in chromosome 18 is not only a marker tumoral transformation, but can predict patient outcome as well (Lu et al., 2015). Although genomic instability events mentioned above were the first discovered to be responsible for Bcl-2 overexpression, they are far from accounting for all cases reported in cancer. Other processes are well documented to lead toward the same results, such as loss endogenous miRNA expression and promoter hypomethylation. For instance, Hanada and colleagues have found that Bcl-2 promoter deficient methylation raised Bcl-2 expression level to a similar or higher level than the t(14;18) chromosomal translocation in chronic lymphocytic leukemia (CLL) (Hanada et al., 1993). In CLL alone, loss of expression of Bcl-2 repressor miR15 and miR16 represents nearly 70% of cases (Calin et al., 2002; Cimmino et al., 2005). Apart from Bcl-2 itself in leukemia, many other malignancies beneficiate from overexpression of an anti-apoptotic Bcl-2 homolog. For example, a global survey conducted on cancer cell lines identified Bcl-2 as the most expressed at the mRNA level in leukemia and lymphoma, Bcl-2A1/Bfl-1 mostly expressed in melanoma whereas Mcl-1 has the highest expression in lung, prostate, breast, ovarian, renal and glioma cancer cell lines (Placzek et al., 2010). On the other hand, there is well documented evidence of pro-apoptotic Bcl-2 family proteins loss of expression in the context of cancer.

For example, the *bax* gene is known to contain a repeated region of 8 guanosines that is very sensitive to base insertion mutations, whenever genomic stability is compromised. Mutations in this region, causing frame-shift mutation, often results in a loss of protein expression or activity. These *bax* mutation have been found in colon cancer and hematopoietic malignancies (Rampino et al., 1997; Meijerink et al., 1998). Loss of tumor suppressor transcription factor p53 found in most cancer, results in the loss of expression of Bax, Bid, Puma and Noxa proteins, which are regulated at post-transcriptional level by p53 (Miyashita et Reed, 1995; Oda et al., 2000; Yu et al., 2003; Sax et al., 2002; Zilfou and Lowe, 2009). In addition, p53 protein might be able to directly regulate Bax activity through the inhibition of the Bcl-2/Bax interaction (Chipuk et al., 2004; Deng et al. 2006).

3.1.1.2. Bcl-2 protein levels define resistance to therapy

We have seen in part 1&2 the role of Bcl-2 proteins in the regulation of programmed cell death both at the mitochondria and the ER. Indeed, most of the cytotoxic gents used to treat cancer induce apoptosis at some point, hence converging on Bcl-2 family regulated pathways. Therefore, anti-apoptotic Bcl-2 homolog levels are often reported to dictate drug response of a tumor cell population (Minn et al., 1995; Simonian et al., 1997). Not only used as a class-defining marker, expression of Bcl-2 proteins such as Bcl-2, Bcl-xL, Mcl-1 or BCL2L10/Nrh are most of the time able to reflect the clinical response of patients to chemotherapy treatments (Bonetti et al., 1998; Nagane et al., 1998; Kaufmann et al., 1998; Cluzeau et al., 2012). Recent advances have shown that it was possible to screen patient tumor cells with BH3-only derived peptides, having selectivity toward their anti-apoptotic preferential partners.

The exposure of tumor cells to a range of Bim, Puma or Bmf BH3 domain peptides leads to specific mitochondrial priming, allowing to deduce on which Bcl-2 homolog a cell population relies for survival (Ni Chonghaile et al., 2011; Montero et al., 2015).

Predicting the dependency of cancer cells on a particular Bcl-2 homolog not only confirm the correlation between expression level and drug resistance, but gives direct access to the combination with the best efficiency for the patient. It gives the opportunity to select the adequate anti-apoptotic Bcl-2 inhibitor for each specific context. Although this approach seems extremely promising, it has three main limitations that still need to be addressed: (1) Inhibition of anti-apoptotic proteins on healthy tissues can lead to severe side effects. For instance, survival of platelets is highly dependent on Bcl-xL, and genetic or pharmacological inactivation is known to cause acute thrombocytopenia (Mason et al., 2007). (2) Inhibition of an anti-apoptotic Bcl-2 homolog only is most of the time not sufficient enough to ensure tumor cells death as it mainly affect steady-state mitochondrial priming. Thereby, reliance on an additional cytotoxic agent is needed, hence increasing possible side effects. (3) Lack of consideration for the protecting effect of anti-apoptotic Bcl-2 homolog at the ER, which is rather BH3-independent. Further investigation is needed to fully integrate both ER and mitochondrial anti-apoptotic functions of Bcl-2 homologs in such strategies.

3.1.2. Bcl-2 protein as players in viral infections

Aside from cancer, emerging roles for apoptosis and the Bcl-2 family have been found in the biology of viral infections. Several DNA viruses such as herpesviruses, adenoviruses have been shown to hijack the apoptotic machinery by expressing their own Bcl-2 homologs, to prolong host cells survival, ensuring a high level of replication and viral particle propagation (Polster et al., 2004). Most of the time, the viral mimicry concerns an anti-apoptotic Bcl-2 homolog, able to neutralize killer proteins Bax or Bak, and BH3 only Bid, Bim or Puma to prevent apoptosis (Kvansakul et Hinds, 2013). Originally, the main viral Bcl-2 proteins described were the adenovirus E1B 19K protein (White et al., 1992; Chiou et al., 1994), Epstein - Barr virus (EBV) BHFR1 protein (Kvansakul et al., 2010), Kaposi sarcoma-associated herpes virus (KSHV) Bcl-2 (Huang et al., 2002). Interestingly, vNr-13a viral homolog of BCL2L10/Nrh has been identified as well, though only in turkey herpesvirus (Aouacheria et al., 2003). More recently, Vaccinia and Variola viruses (Kvansakul et al., 2007; 2008), as well as Cytomegaloviruses (Ma et al., 2012) have also been reported to express viral Bcl-2 homologs.

Beside their assisting role in viral infections, expression of these viral proteins could be linked in some case to infected cells transformation into neoplastic lesions. For instance, EBV BHFR1 protein was shown to participate in lymphomagenesis (Kelly et al., 2009).

Thereby, viral Bcl-2 homologs could be interesting targets by offering new therapeutic opportunities in infectious diseases. This approach may offer distinctive advantages such as increased specificity and limited probable side effects compared to the pharmacological inhibition of endogenous Bcl-2 aberrantly expressed in cancer.

3.2. Targeting Bcl-2 proteins activity

Due to their involvement in disease and that their action mechanism properly characterized, a number of strategies have been devised to inhibit Bcl-2 proteins action. Mainly formulated as small organic molecules to optimize their bioavailability and pharmacokinetic properties, Bcl-2 inhibitors could nevertheless be active as peptides, trading in that case increased specificity for poor cell penetration ability.

Accordingly, compared to the enhanced stability of small organic molecules, peptides are prone to proteolytic degradation. This low stability can also confer some advantages, as peptides breakdown does not generate toxic degradation products. Bcl-2 proteins inhibitors are for the most part mimicking a specific BH3 domain to either antagonize anti-apoptotic members or activate pro-apoptotic members. This main class is thus called "BH3 mimetics" from its inclination to imitate structurally and functionally BH3 domain of BH3-only protein. In addition, a last class of agents is also known to convert anti-apoptotic proteins into pro-apoptotic killers.

We will review these different strategies in the part below (See Table 3).

3.2.1. Antagonists of pro-survival proteins

3.2.1.1. Small organic molecules

3.2.1.1.1. Pan-Bcl-2 proteins inhibitors 3.2.1.1.1.1. Obatoclax

Obatoclax is a synthetic molecule derived from the natural product prodigiosin. It behaves as a nonspecific anti-apoptotic Bcl-2 inhibitor, binding the hydrophobic groove of all pro-survival Bcl-2 family members with a low affinity (>1 μ M). Although it has demonstrated a potent activity *in vitro* (Nguyen et al., 2007) and was one of the first Bcl-2 inhibitor molecules to be approved for clinical trial for cancer, it only showed a marginal effect on patients (Hwang et al., 2010). Obatoclax was later shown to have an indirect activity on apoptosis, triggering Noxa transcriptional up-regulation through an ER-stress pathway rather than directly inhibiting pro-survival Bcl-2 proteins (Albershardt et al., 2011).

3.2.1.1.1.2. Gossypol family

AT-101, a synthetically produced isomer of Gossypol natural polyphenol, has shown pro-survival Bcl-2 inhibition properties. Like obatoclax, it has shown a good activity *in vitro* ($0.2 < K_d < 1 \mu$ M), but a limited anti-tumoral activity in clinical trials. (Paoluzzi et al., 2008; Baggstrom et al., 2011; Stein et al., 2016). Several studies have shown that AT-101 might trigger simultaneously apoptosis and a compensating cytoprotective autophagy, the latter linked with chemoresistance (Mani et al., 2015). However, some studies differs in the mechanism involved. In some cases, AT-101 was shown to possibly involve disruption of the Bcl-2/Beclin-1 interaction, whereas in MCF7 autophagy triggered by AT-101 was shown to be Bcl-2 and Bcl-xL independent (Lian et al., 2011; Antonietti et al., 2016). Gossypol second generation derivatives TW-37 and BI-97C1 have shown a more promising activity, with a moderate affinity for Bcl-2, Bcl-xL ($0.2 < K_d < 1 \mu$ M), and might target slightly more preferentially Mcl-1 (Wang et al., 2006; Mohammad et al., 2007; Wei et al., 2010; Dash et al., 2011). Although these inhibitors have not been clinically tested, further development of these molecules toward increasing selectivity and affinity for Mcl-1, especially for TW-37, seems feasible and might be worthwhile to consider (Varadarajan et al., 2013).

3.2.1.1.1.3. *ABT-737 and derivatives*

Purely designed by NMR-mediated SAR (Structure-Affinity Relationship), ABT-737 is the first high affinity (<100 nM) inhibitor for Bcl-2, Bcl-xL and Bcl-W but not Mcl-1 or Bfl-1/Bcl-2A1 (Oltersdorf et al., 2005). ABT-737 is therefore referred to as a BH3 mimetic "Bad-like" from its specificity range toward pro-survival proteins.

ABT-737 has been further developed into an orally bioavailable drug, ABT-263 (Navitoclax), ideal for clinical use (Tse et al., 2008). Numerous well documented clinical studies have shown ABT-263 antitumoral efficiency in leukemia, although patient response rate achieved is usually between 20 to 30% (Wilson et al., 2010; Roberts et al., 2012). In contrast, in solid tumors, several studies have not shown any patient response, but in the best case stable disease for some patients (Rudin et al., 2012; Cleary et al., 2014). Indeed, limitations have soon appeared: (1) Platelets survival are highly dependent on Bcl-xL activity, and thrombocytopenia was a problematic dose-limiting side effect both in mice and humans for ABT-737 (Mason et al., 2007). Increased efforts are to be made to ensure more specific inhibition of Bcl-2 pro-survival proteins. (2) Resistance to ABT-737 treatment is in most case mediated by Mcl-1 protein, which is not an ABT-737 target. Therefore, efficient apoptosis priming might require additional inhibition of Mcl-1 (van Delft et al., 2006; Konopleva et al., 2006; Chen et al., 2007). Finally, some "Bad-like" derivatives from ABT-737 were optimized for increased affinity for Bcl-2 and

Bal-xL (($K_d < 1$ nM). These molecules, namely BM-957 and BM-1197, have shown a better effect on tumor regression than ABT-737 in xenograft mice models, while still suffering from ABT-737 liabilities i.e. platelets depletion.

3.2.1.1.2. Specific inhibitors

After seeing that broad spectrum inhibition anti-apoptotic Bcl-2 proteins may carry some limitations, it became obvious to dispose of several highly specific inhibitors for each Bcl-2 pro-survival proteins. We will list below the main molecules designed in recent years to inhibit only one pro-survival Bcl-2, and discuss their activity.

3.2.1.1.2.1. ABT-199

ABT-199 (Venetoclax) has been specifically designed as a pure Bcl-2 inhibitor, to circumvent ABT-737 inherent limitations i.e. inhibition of Bcl-xL and Bcl-W linked with side effects on platelets. ABT-199 displays an impressive affinity ($K_i < 0.010$ nM) for Bcl-2 in contrast to a 48 nM K_i for Bcl-xL (Souers et al., 2013). In addition, it has shown a potent effect in xenografted mice model of tumor growth while sparing platelets. One of the latest clinical phase I study using ABT-199 has shown 20% percent of Chronic Lymphocytic Leukemia (CLL) patients in complete remission, with a response rate of 79% overall (Roberts et al., 2016). Another phase II study has shown similar response rate in treatment refractory CLL patients (Stilgenbauer et al., 2016). To date, ABT-199 is the only phase III approved and most promising anti-Bcl-2 molecule.

3.2.1.1.2.2. WEHI-539

Discovered from a high-throughput screening followed by structure-guided development, WEHI-539 and its refined counterpart A-1155463 are the most potent specific Bcl-xL inhibitors known (Lessene et al., 2013; Tao et al., 2014).

WEHI-539 has shown a 1 nM K_d for Bcl-xL, slightly higher than Bim-BH3 peptide (K_d = 0.2 nM), and can trigger both fibroblast and platelets Bax/Bak-dependent apoptosis *in vitro*. A-1155463 displays an even better affinity for Bcl-xL (K_d < 0.010 nM), while exhibiting better pharmacokinetic properties. A single injection of A-1155463 (5 mg/kg) was shown to induce a transient platelets depletion in mice (within 6 hours), similarly to ABT-263 (Tse et al., 2008).

However, as platelets depletion is a strong drawback, and the effect on tumor growth is somewhat limited, use of these inhibitors may be restricted to research use rather than therapeutic applications.

3.2.1.1.2.3. Mcl-1 inhibitors

Specific inhibitors have been designed to target Mcl-1, especially since Mcl-1 overexpression is a known mechanism for tumor cell resistance from ABT-737. Most of these inhibitors have been classified as "Noxa-like", from their structural analogy to Noxa BH3 domain.

Many inhibitors have been designed very recently, and few have been properly characterized as extensively as for other inhibitors (Friberg et al., 2013; Bruncko et al., 2015).

Nevertheless, we can quote among them the molecule A-1210477, which binds to Mcl-1 with a K_d of 0.45 nM, and synergizes with ABT-263 to induce apoptosis in a wide array of cancer cell lines (Leverson et al., 2015). A-1210477 is thus the only BH3-mimetic with a high selectivity toward Mcl-1, having a proven biological activity. This molecule and its further refinements could be very interesting tools to forestall ABT-199 resistance, mainly defined by Bcl-xL and Mcl-1 overexpression (Lin et al., 2016).

3.2.1.1.2.4. ML258

ML258 is a BH3 mimetic compound which has been developed as a specific inhibitor of the Bim:BCL2L10/Nrh interaction. It showed a K_d of 386 nM for BCL2L10, whereas it has a low affinity for Bcl-xL ($K_d > 50 \mu$ M).

Nonetheless, it failed to achieve any significant effect in cell lines overexpressing BCL2L10/Nrh, as well as potentiating staurosporine-mediated apoptosis (Zhou et al., 2011).

It is not known whether ML258 is not active through lack of specificity or whether BCL2L10/Nrh mechanism of action does not require interaction with Bim to protect from apoptosis.

3.2.1.2. Peptides

3.2.1.2.1. SAHB peptides

SAHB peptides, standing for Stabilized Alpha-Helix of Bcl-2 domain, is a strategy used by Walensky and colleagues to chemically modify BH3 peptides, improving their pharmacokinetic properties. This strategy, referred as "hydrocarbon stapling", was shown to promote helical conformation, decrease protease-mediated degradation, increase cell permeability and affinity for pro-survival Bcl-2 proteins hydrophobic groove (Walensky et al., 2004).

Bid-BH3 SAHB peptide was shown to bind Bcl-2 with a six-fold higher affinity than unmodified peptide (39 nM and 269 nM respective K_d), and was able to induce tumor cell apoptosis in a mice xenograft model. Bim-BH3 SAHB peptide has been shown to bind and inhibit Bcl-xL, Bcl-W, Mcl-1 and Bfl-1/Bcl-2A1 (Labelle et al., 2012). This peptide was also reported to induce apoptosis in leukemia cell lines, as well as tumor regression in human Acute Myeloid Leukemia xenograft mice model (AML). In another study, it was shown that Mcl-1 own BH3 domain could be used to make a Mcl-1 inhibitory BH3 SAHB peptide (Stewart et al., 2010). However, a recent study has questioned the activity of SAHB peptides, especially in regard to their enhanced binding affinity and cell permeability (Okamoto et al., 2013). Therefore, caution should be used with such system, as position of the staples has a critical impact on peptide biological activities (Bird et al., 2014).

3.2.1.2.2. NOXA-derived BH3 peptides

Biphenyl-cross-linked Noxa peptide has been use to specifically bind Mcl-1 with a K_d of 3.4 nM, similar to unmodified Noxa peptide ($K_d = 6.7$ nM), wheras affinity for Bcl-xL was reported to be much lower ($K_d > 1000$ nM). Chemical crosslinking was shown to increase Noxa peptide helical conformation and cell permeability, but also increased resistance to proteolysis (Muppidi et al., 2012). Furthermore, addition of an ubiquitin chain allowed an enhanced internalization of the Noxa peptide through the CXCR4 receptor, enhancing its killing activity in cell lines (Muppidi et al., 2014).

3.2.1.2.3. IP3R1 BIRD-2 peptide

Few strategies have been formulated to inhibit Bcl-2 proteins actions apart from the canonical interaction between BH3 domains and the hydrophobic groove of pro-survival proteins. One of them consists in using a short peptide derived from the interaction site of Bcl-2 on IP3R1 suppress the cytoprotective function of Bcl-2 BH4 domain. Indeed, Distelhorst and his colleagues have shown that such peptide, is able to disrupt specifically the Bcl-2/IP3R1 interaction, but not Bcl-2/Bim interaction (Rong et al., 2008; 2009).

This peptide, called BIRD-2 standing for Bcl-2 IP3 Receptor Disruptor-2, has been shown to sensitize to calcium-driven apoptosis by lifting Bcl-2 inhibition on IP3R-mediated calcium signaling. To note, this BIRD-2 peptide encompasses a cell penetrating domain derived from the HIV TAT peptide sequence to allow permeability across biological membranes (Bechara et Sagan, 2013).

Accordingly, it has been demonstrated that BIRD-2 treatment induces calcium-driven apoptosis in primary CLL tumor cells, but not in healthy ones (Zhong et al., 2011). In Diffuse Large B-Cell Lymphoma (DL-BCL) cell lines, it has been found that IP3R2 receptor expression level correlates with an apoptotic response to BIRD-2 peptide (Akl et al., 2013). Cell lines having none or a weak expression of IP3R2 were resistant to BIRD-2 treatment.

This result demonstrates that tumor cells could be addicted to Bcl-2 to suppress pro-apoptotic IP3R2 calcium signaling, and suggests that Bcl-2 inhibition on calcium fluxes should be preferentially exerted at the IP3R2 receptor in lymphomas. Finally, BIRD-2 peptide activity has also been demonstrated recently in human myeloma and small-cell lung cancer (SCLC) cell lines, and is further enhanced by combination with ABT-263 or ABT-199 (Lavik et al., 2015; Greenberg et al., 2015). These data showed that targeting Bcl-2 BH4 with BIRD-2 is a complementary approach from traditional BH3 mimetic, and combination of both would elicit the best response in cancer treatment.

3.2.2. Activators of pro-apoptotic Bcl-2 proteins

Aside from inhibition of pro-survival proteins, some studies have laid the proof-of-concept that direct activation of pro-apoptotic executor proteins Bax and Bak might be feasible. This approach is currently an early concept, which may provide excellent experimental tools, but may not be relevant as a therapeutic strategy yet.

3.2.2.1. BIM-derived

Bim BH3 SAHB staple peptides have been reported to directly activate Bax, in addition to inhibit prosurvival Bcl-2 homologs (Gavathiothis et al., 2008; 2010). From the interface between Bim BH3 SAHB peptide and Bax, a small organic molecule called BAM-7 has been rationally designed as a direct Bax activator (Gavathiothis et al., 2012).

BAM-7 has a K_d of 3.3 μ M for Bax, compared to the K_d of 283 nM of Bim BH3 SAHB for Bax. This is due to a smaller binding interface compared to the Bim BH3 peptide. Accordingly, BAM-7 is able to trigger a Bax dependent apoptosis, and has no effect on Bax knock-out cells. Moreover, BAM-7 does not interact with Bax BH3 binding pocket, nor with Bak.

3.2.2.2. PUMA-derived

Bak activation is known to be dependent on a transient binding of an activator BH3 domain into Bak BH3 binding groove (Dai et al., 2011). Therefore, it was thought that using a BH3 domain, Bak activation could be triggered. Indeed, Puma BH3 domain was assessed as a direct Bak activator. It was shown to bind Bak BH3 binding pocket with an affinity of 26 nM, induce Bak oligomerization and apoptosis (Dai et al., 2014).

Puma BH3 SAHB peptides have also been designed following the Walensky group "staple peptides" concept, and have been shown to bind Bax at two different sites: a Bax canonical BH3 binding site, and a non-canonical "trigger" site (K_d of 70 nM and 221 nM respectively) (Edwards et al., 2013). Interestingly, affinity was three times higher for the non-canonical site, suggesting as for BAM-7 that Puma might activate Bax with a distinct mechanism from Bak.

3.2.3. Pro-survival converting agents

In this last part, we will discuss an amazing class of agents, peptides and proteins, which does not inhibit pro-survival Bcl-2 proteins, but instead convert them into pro-apopotic molecules. This clever way of counteracting Bcl-2 protein action would reside on putative conformation changes upon binding of these modulators, and should be a promising strategy to develop in the future.

3.2.3.1. Nur77-derived peptides

Nur77/NR4A1 is a DNA binding protein, originally described as an orphan nuclear receptor transcription factor, and is a known effector of T-Cell Receptor (TCR) apoptotic response in T lymphocytes (Yazdanbakhsh et al., 1995). Inhibition of Nur77 has been described to impair apoptosis execution, and allows the survival of self-reactive T-cells that should be eliminated in normal conditions (Zhou et al., 1996).

More recently, several studies have shown a direct involvement of Nur77 in apoptosis, as it seemed able to modulate directly the activity of several anti-apoptotic Bcl-2 proteins. Nur77 deleted from its DNA binding domain was shown to interact with Bcl-2 N-terminal region, within the loop between BH4 and BH3 domains (Lin et al., 2004).

Conformational rearrangement of Bcl-2 occurs after Nur77 binding, exposing Bcl-2 BH3 domain, which is otherwise buried into Bcl-2 core structure. Having its BH3 exposed in such manner, it has been demonstrated that Bcl-2 behaves as a pro-apoptotic sensitizer BH3-only protein to trigger Bax/Bak-dependent mitochondrial cell death.

Furthermore, a 9-amino acids peptide from Nur77 conjugated with a polyarginine cell penetrating domain, called NuBCP-9, was shown to conserve the same Bcl-2 converting properties (Kolluri et al., 2008).

NuBCP-9 peptide and its protease resistant enantiomer were shown to bind Bcl-2 with a K_d of 1.7 μ M and 2.5 μ M respectively. Interestingly, Bcl-2A1/Bfl-1 and BCL2L10/Nrh were also found to interact with NuBCP-9, the interaction with BCL2L10/Nrh being the strongest (K_d = 20 nM), but not Bcl-xL, Bcl-W and Mcl-1 (Luciano et al., 2007; Kolluri et al., 2008). A common interaction interface between Bcl-2 family proteins and NuBCP-9 has been recently described, confirming that the binding site for NuBCP-9 is not the canonical BH3 binding pocket (Godoi et al., 2016).

Finally, NuBCP-9 peptide was shown to induce Bcl-2 or BCL2L10/Nrh-dependent apoptosis in myeloma and breast cancer cells. However, it has been found that interaction between the cargo (Nur77 peptide) and the cell penetrating moiety (polyarginine sequence) of NuBCP-9 was causing non-specific plasma membrane disruption and necrosis at high concentrations (Watkins et al., 2011). New strategies have been recently devised to enhance Nur77-derived peptide non-toxic uptake in tumor cells, such as PolyEthylen Glycol (PEG)/ polylactic acid encapsidation (Kumar et al., 2014).

3.2.3.2. BDA-366

A virtual screening of ~300 000 small organic molecules against Bcl-2 BH4 domain allowed the identification of the compound BDA-366. This molecule has shown a potent anti-tumor activity on both SCLC and non-SCLC cancer cell lines expressing Bcl-2, while Bcl-xL and Mcl-1 expression do not seem to correlate with BDA-366 effectiveness (Han et al., 2015). Binding of BDA-366 to Bcl-2 BH4 ($K_d = 3.3$ nM) induced a conformational change that caused exposure of its BH3 domain, but unlike Nur77 peptide it was reported that this rearrangement leads to Bax direct activation.

Amazingly, BDA-366 was also found to disrupt Bcl-2/IP3R interaction, and enhance IP3R-mediated calcium release. BDA-366 and RAD001 mTOR inhibitor used together have shown an impressive effect and induced tumor regression in a mice model engrafted with lung cancer cell line.

At last, BDA-366 displayed a striking effect on multiple myeloma primary tumor cells, able to trigger apoptosis in myeloma but not in normal mononuclear cells (Deng et al., 2016). These results were confirmed in myeloma mice models, where BDA-366 was shown to suppress tumor cell growth. Interestingly, BDA-366 was reported to decrease Bcl-2 S70 phosphorylation, although no underlying mechanism has been found yet.

Compound	Source/ Identification	Targets	Mechanism	Affinity (K _d)	Bax/Bak dependent apoptosis	Ca ²⁺ driven apoptosis	Antitumor effect in vivo	Clinical data/efficiency
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Antagonists	of	pro-survival	proteins
rintagomoto	•••	pro our rivur	procenie

Obatoclax	Natural product derived	Bcl-2 Bcl-xL Bcl-W Mcl-1 Bfl-1 Nrh	pan-BH3 mimetic	>1 µM	No	ND	Yes	Yes/No
AT-101	Gossypol derivative	Bcl-2 Bcl-xL Bcl-W Mcl-1 Bfl-1 Nrh	pan-BH3 mimetic	0.2 <k<sub>d<1µM</k<sub>	Partial	ND	Yes	Yes/No
TW-37	Gossypol derivative	Bcl-2 Bcl-xL Mcl-1	pan-BH3 mimetic with increased specificity to Mcl-1	0.2 <k<sub>d<1µM</k<sub>	Yes	ND	Yes	
BI-97C1	Gossypol derivative	Bcl-2 Bcl-xL Mcl-1	pan-BH3 mimetic	0.2 <k<sub>d<1µM</k<sub>	Yes	ND	Yes	
ABT-737/263	NMR/SAR rational design	Bcl-2 Bcl-xL Bcl-W	Bad-like BH3 mimetic	<100 nM	Yes	ND	Yes	Yes/Yes
ABT-199	SAR optimisation of ABT-263	Bcl-2	Bcl-2 specific BH3 mimetic	<0.01 nM	Yes	ND	Yes	Yes/Yes
WEHI-539	High throughput SOM screen	Bcl-xL	Bcl-xL specific BH3 mimetic	<1 nM	Yes	ND	ND	
A-1155463	SAR optimisation of WEHI-539	Bcl-xL	Bcl-xL specific BH3 mimetic	<0.01 nM	Yes	ND	Yes	
A-1210477	SAR rational design	Mcl-1	Mcl-1 specific BH3 mimetic	0.45 nM	Yes	ND	ND	
ML258	High throughput SOM screen	Nrh	Nrh specific BH3 mimetic	386 nM	Yes	ND	No	
Bid-SAHB	Bid BH3 stapled peptide	Bcl-2 ?	Bid BH3 mimetic	39 nM	Yes	ND	ND	
Bim-SAHB	Bim BH3 stapled peptide	Bcl-2 Bcl-xL Mcl-1 Bfl-1	Bim BH3 mimetic	2 <k<sub>d<10nM</k<sub>	Yes	ND	Yes	
Noxa peptide	Noxa BH3 Biphenyl- crosslinked peptide	Mcl-1	Noxa BH3 mimetic	3.4 nM	Yes	ND	ND	
BIRD-2	IP3R1- derived peptide	Bcl-2	Disruptor of IP3Rs/Bcl-2 interaction	~1 µM	No	Yes	Yes	

Activators of pro-apoptotic proteins

BAM-7	SAR design from Bim BH3	Bax	Triggers Bax conformational activaion	3.3 µM	Yes	ND	ND	
Puma-SAHB	Puma BH3 stapled peptide	Bax Bak	Binds to Bak hydrophobic groove/ Activate Bax	26 nM (Bak) 70 nM (Bax)	Yes	ND	ND	

Pro-survival converting agents

Nur77 peptide	Nur77 derived peptide	Bcl-2 Bfl-1 Nrh	Convert Bcl-2/Bfl-1/Nrh into a BH3-only pro-apoptotic protein	20 nM (Nrh) 1.7 μM (Bcl-2) ND (Bfl-1)	Yes	ND	Yes	
BDA-366	High throughput SOM virtual screen	Bcl-2	Convert Bcl-2 into a BH3-only pro-apoptotic protein	3.3 nM	Yes	ND	Yes	

ND: Not Determined

SAR: Structure-Affinity Relationship SOM: Small Organic Molecule SAHB: Stabilized Alpha-Helix of Bcl-2 domain

Table 3 - Anti-Bcl-2 strategies

4. BCL2L10/Nrh, the outsider of the Bcl-2 protein family

Among the Bcl-2 pro-survival proteins, Bcl-B/BCL2L10/Nrh is one of the least studied. On one hand, biochemical characterization of this protein is quite good, as we know for instance its interaction pattern with Bcl-2 family members (Rautureau et al., 2012), and that it is involved in autophagy through its interaction with Beclin-1 (Robert et al., 2012).

Even its 3-dimensional protein structure was determined, as it was guessed that BCL2L10/Nrh might be a protein of important value in cell physiology, by analogy with the other pro-survival Bcl-2 homologs. On the other hand, physiological and pathological function of BCL2L10/Nrh until very recently have been poorly known, and are barely beginning to be understood.

4.1. BCL2L10/Nrh involvement in pathologies

The disappointing results of Nrh mouse ortholog knock-out were interpreted as a lack of physiological impact of the protein (Russell et al., 2002). Indeed, it was mentioned in an article by Reed and colleagues that Nrh protein expression was seldom detected in healthy tissues (Krajewska et al., 2008).

We sought to revisit this result, and we confirmed this previous finding (see Article – 2 Complementary Figure 1). Nrh was only detected strongly expressed in the normal kidney, with a moderate expression observed in cerebral cortex, cerebellum, fallopian tube, esophagus, thyroid and weak expression in liver tissue. An immunostaining against Nrh was used, as it was feared that mRNA detection would not be as accurate, considering the studies showing post-translational regulation of Nrh expression level by the ubiquitin/proteasome pathway (Beverly et al., 2012; van de Kooij et al., 2013). Although expression in most healthy tissue proved very low, the same study from Reed and colleagues have found that Nrh is a marker of bad prognosis in various neoplastic disorders. For example, Nrh expression was found highly expressed in *in situ* breast carcinomas and even more so in invasive breast tumors, but not adjacent epithelial tissues (Krajewska et al., 2008).

A high expression was also the hallmark of advanced prostate cancer in this study, as well as allowing to discriminate between good and bad prognosis groups for Small Cell Lung Cancer (SCLC) (See **Figure 17**).



D'après Cluzeau et al., 2012

Figure 17 - BCL2L10/Nrh involvement in pathologies

Figure 17 - BCL2L10/Nrh involvement in pathologies

The Bcl-2 pro-survival homolog BCL2L10/Nrh (called Bcl-B in study by Krajewska and colleagues) is known to be involved in tumor progression.

(a) Bcl-B expression assessed by ImmunoHistoChemistry (IHC) in breast cancer patients (n=119), with expression level below detection level in adjacent normal mammary epithelium (NE), with a prevalence of 64% in *in situ* breast carcinoma (IS) and 89% in invasive cancer (INV) (Krajewska et al., 2008).

(b) Bcl-B expression in Small Cell Lung Cancer (SCLC) patients (n=79) was assessed by IHC and high expressing Bcl-B tumors (based on median) were linked with shorter overall survival than low expressing Bcl-B tumors.

(c) BCL2L10 expression detected by flow cytometry in Acute Myeloid Leukemia and Myelodysplatic Syndroms patients showed that high expression of BCL2L10 is correlated with resistant to chemotherapy treatment (Azacytidine).

Results from the same study suggested that in gastric cancer only, Nrh would be instead a good prognosis marker. This was confirmed afterwards by several studies showing that hypermethylation silencing of Nrh gene promoter was a bad prognosis discriminant in gastric carcinoma (Xu et al., 2010; Mikata et al., 2010). Interestingly it was also reported that Nrh silencing increased tumor cell growth, which can corroborate with the reduced proliferation induced by BCL2L10/Nrh overexpression in yeast (Aouacheria et al., 2001; Xu et al., 2011). To summarize, Nrh seems to act in a different manner in gastric cancer than in other organs neoplastic lesions.

Aside from solid tumors, expression of BCL2L10/Nrh has been detected in myelodysplatic syndroms (MDS) and acute myeloid leukemia (AML) (Cluzeau et al., 2012). Percentage of more than 50% tumor cells stained for BCL2L10 was linked with a much shorter overall survival than patient with low BCL2L10 expression. Not only accounting for the overall survival of patients, BCL2L10 high expression level in tumor cells correlated with resistance to Azacytidine, a demethylating chemotherapy agent (See Figure 17).

Futhermore, it has been reported recently that loss of BCL2L10 homozygosity from a chromosome translocation could be the leading cause of inherited affective psychosis disorder (Bouwkamp et al., 2016). Finally, these findings clearly establish a role for BCL2L10/Nrh, mostly in cancer progression and resistance to chemotherapy.



Figure 18 - BCL2L10/Nrh ortholog Nrz invalidation in zebrafish

Figure 18 – BCL2L10/Nrh invalidation in zebrafish

BCL2L10/Nrh ortholog Nrz, displays a lethal phenotype when invalidated in zebrafish.

(a) During zebrafish gastrulation stage, the cells migrate down the embryo, from the animal pole to the vegetal pole in a process called Epiboly. Whenever Nrz is invalidated through an anti-sens Morpholino, the phenotype is characterized by a developmental arrest at 40% Epiboly. An early constriction within a contractile tissue called Yolk Syncytial Layer (YSL) happens, leading ultimately to embryo cells, the blastomeres, to detach from the yolk and the embryonic death. In normal developing embryos, the actin myosin ring typically forms around 75% Epiboly, instead of 40 % for Nrz knocked-down embryos.

(b) Blocking Nrz activity leads to uncontrolled calcium fluxes within the embryo and the formation of the Actin-Myosin ring too soon. Nrz was shown to prevent calcium-dependent CAMKIIβ activation, which in turn can activate MCLK (Myosin Light Chain Kinase) by phosphorylation, triggering this "muscle-like" contraction and leading to the lethal phenotype.

4.2. Linking biochemistry with the physiological function of BCL2L10/Nrh

Amazingly, it is not the mammalian model that provided us with the more clues about BCL2L10/Nrh function, but the invalidation of its zebrafish counterpart, Nrz. Knock-down of Nrz expression in zebrafish embryo, using anti-sens phosphorodiamidate morpholino oligomer (referred as morpholino), shown a lethal phenotype specifically during the gastrulation developmental stage (Arnaud et al., 2006). During gastrulation in zebrafish, cells migrate from the animal pole (top) to the vegetal pole (bottom) of the embryo, in a process called Epiboly. The muscle-type contraction of an actin-myosin ring within a specialized embryonic tissue, called the Yolk Syncytial Layer (YSL), occurs at 75% of epiboly to achieve this process.

Nrz invalidation was shown to induce excessive calcium transient within the YSL. Interestingly, this aberrant calcium release do not seem to affect the mitochondria, as caspase activation was not reported. Abnormal calcium signals were shown instead to trigger the actin-myosin ring too soon, at 40% of epiboly in place of 75%, literally ripping the embryos apart (Popgeorgiev et al., 2011).

Popgeorgiev and colleagues demonstrated that Nrz was able to suppress IP3R1-mediated calcium signaling by directly interacting with this receptor via its BH4 domain (See **Figure 18**). These new findings have left nonetheless unanswered questions, such as the precise biochemical characteristics of this novel IP3R1/Nrz interaction. Moreover, the existence of an ON/OFF switch for calcium signals was suggested, as calcium transient are still required to coordinate the actin myosin ring assembly at some point, to complete embryogenesis. This last point could involve post-translational modifications of Nrz, as we know for instance that phosphorylation can often regulate Bcl-2 proteins activity.

Finally, this interaction between Nrz and IP3R1 pose a fundamental question: does this interaction exist in human? Could it contribute to suppress calcium-driven apoptosis in the same manner as Bcl-2 interaction with IP3Rs in tumor cells?

RESULTS

Article 1 - Lessons learnt from Nrz function during zebrafish development

Introduction to Article 1

In the following work, we sought to understand the molecular mechanism explaining Nrz ability to suppress IP3R1-mediated calcium signaling as demonstrated in a previous study from our team (Popgeorgiev et al., 2011). To that end, we cloned the zebrafish IP3R1 receptor (noted zIP3R1) divided into six functional domains, from mouse IP3R1 primary sequence organization. This division of IP3R1 in smaller domains would allow to locate Nrz interaction site within IP3R1. We also used different Nrz deletion mutants, to investigate the minimal domain required for interaction with IP3R1 and calcium regulation. These mutants could be used both for the biochemical characterization of the interaction, and the functional complementation of Nrz MO embryo with *in vitro* synthetized mRNAs. Moreover, some Nrz deletion mutants having lost their canonical BH3 binding pocket, would be further used to demonstrate whether BH3 binding ability is a prerequisite for Nrz control on calcium signaling.

Finally, as we suspected that Nrz activity to suppress calcium signaling would likely to be dynamically regulated, we investigated Nrz phosphorylation status during zebrafish embryogenesis. Indeed, Nrz phosphorylation was detected during epiboly, and we generated constitutive phosphorylation absence or phosphorylation mimicking mutants (from Ser&Thr to Ala and Glu respectively). It was shown that phosphorylation of key residues within Bcl-2 inter BH4-BH3 domain loop control its ability to suppress calcium signals (Bassik et al., 2004). Therefore, we investigated if similar mutations could affect Nrz function in a same way, both in human cells and in zebrafish embryos.

The Bcl-2 Homolog Nrz Inhibits Binding of IP₃ to Its Receptor to Control Calcium Signaling During Zebrafish Epiboly

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One Sentence Summary: Phosphorylation of a Bcl-2-like protein allows calcium signals needed for the early stages of development.

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Abstract

Members of the Bcl-2 protein family regulate mitochondrial membrane permeability and also localize to the endoplasmic reticulum where they control Ca2+ homeostasis by interacting with inositol 1, 4, 5-trisphosphate receptors (IP3Rs). In zebrafish Bcl-2-like 10 (Nrz) is required for Ca2+ signaling during epiboly and gastrulation. Here, we characterized the mechanism by which Nrz controls IP3-mediated Ca2+ release during this process. We showed that Nrz was phosphorylated during early epiboly and that in embryos in which Nrz was knocked down, reconstitution with Nrz bearing mutations designed to prevent its phosphorylation disrupted cyclic Ca2+ transients and the assembly of the actin-myosin ring and led to epiboly arrest. In cultured cells, wild-type Nrz, but not Nrz with phosphomimetic mutations, interacted with the IP3 binding domain of IP3R1, inhibited binding of IP3 to IP3R1, and prevented histamine-induced increases in cytosolic Ca2+. Collectively, these data suggest that Nrz phosphorylation is necessary for the generation of IP3-mediated Ca2+ transients and the formation of circumferential actin-myosin cables required for epiboly. Thus, in addition to their role in apoptosis, by tightly regulating Ca2+ signaling, Bcl-2 family members participate in the cellular events associated with early vertebrate development, including cytoskeletal dynamics and cell movement.

Introduction

The Bcl-2 protein family has both pro- and anti-apoptotic functions, including the control of mitochondrial membrane permeability to cytochrome c, which leads to caspase activation (1). In addition, Bcl-2 proteins localize to the endoplasmic reticulum (ER) where they participate in Ca^{2+} homeostasis by binding to the Ca^{2+} channel, inositol 1, 4, 5-trisphosphate (IP₃) receptor (IP₃R) (2). IP₃ binding to IP₃Rs promotes Ca^{2+} efflux from the ER into the cytosol, a process known as IP₃-induced Ca^{2+} release (IICR). IP₃Rs are composed of five types of domains (3): the suppressor domain (SD), the IP₃-binding domain (IP₃BD), the modulatory and transducing domain (MTD), the channel forming domain (CFD), and the coupling domain (CD). The SD and IP₃BD mediate binding to IP₃, the MTD binds regulatory molecules that modify IP₃R activity and transduces the signal from the IP₃BD to the CFD, and the CD is implicated in IP₃R tetramerization and channel opening (4). Bcl-2 inhibits IICR by interacting with the MTD (5), whereas Bcl-xL promotes Ca^{2+} release at low concentrations of IP₃ by interacting with the CD (6).

 Ca^{2+} release through IP₃Rs mediates many cellular and physiological processes, such as cell proliferation, differentiation, apoptosis, fertilization, and embryonic development (7). In the zebrafish embryo, Ca^{2+} signaling plays a role in early stages of development (8). At the onset of the blastula stage, blastomeres that are in contact with the yolk release the contents of their cytoplasm into the yolk cell forming the yolk syncytial layer (YSL).

At the end of blastulation, the embryo comprises blastomeres on top of the yolk cell, which contains the YSL. Prior to gastrulation, the blastomeres and the YSL begin to migrate from the animal to vegetal pole in a process known as epiboly (9). In the YSL, IP₃R-dependent Ca²⁺ signaling occurs at the onset of epiboly (10) and again once the blastomeres have passed the equator of the embryo (11). A contractile actin-myosin ring forms at the blastoderm margin (12) and is likely required for epiboly progression.

We recently demonstrated that the anti-apoptotic protein Nrz, the zebrafish ortholog of Nrh-Bcl2l10, plays a crucial role in the progression of zebrafish epiboly. During early development, zygotic *nrz* is specifically expressed in the YSL and knockdown of Nrz by morpholino (MO) injection results in epiboly arrest, constriction of the blastoderm margin, and detachment of the embryo from the yolk cell (13). This phenotype does not require caspase activity; instead, Nrz binds to IP₃R1 on the surface of the ER and decreases IICR (14). Knockdown of *nrz* increases the concentration of Ca²⁺ at the blastoderm margin and promotes phosphorylation of myosin light chain (MLC) and premature formation of the circumferential actin-myosin ring resulting in the detachment of blastomeres from the yolk (14).

Here, we investigated how Nrz regulates Ca^{2+} signals in the YSL during epiboly. We demonstrated that Nrz interacted with the IP₃-binding domain of IP₃R1 and disrupted its binding to IP₃. Furthermore, we showed that phosphorylation of Nrz prevented this interaction and promoted Ca^{2+} oscillations and formation of the actin-myosin ring during epiboly.

Results

The BH4-BH1 region of Nrz is required to inhibit IP₃-induced Ca²⁺ release

We previously demonstrated that Nrz interacts with IP₃R1 via its N-terminal Bcl-2 homology (BH) 4 domain (14). Other members of the Bcl-2 family, including Bcl-2 and Bcl-xL, interact with IP₃R1 (5, 6) and control IICR. Although the BH4 domain of Bcl-2 is sufficient to suppress IICR (15), the BH4 domain of Bcl-xL is not (16). To ask whether the BH4 domain of Nrz was required for IICR, we fused the BH4 domain of Nrz to the ER-targeting sequence of cytochrome b5 (NrzBH4Cb5) (Fig. 1A) and transiently overexpressed the fusion protein in HeLa cells. Similar to the Nrz BH4 domain that localizes in the cytoplasm (14) or full-length Nrz targeted to the ER (NrzCb5), NrzBH4Cb5 co-immunoprecipitated with endogenous IP₃R1 (Fig. 1B). To assess whether NrzBH4Cb5 affects IICR we monitored changes in the concentration of cytosolic Ca²⁺ using a Ca²⁺-sensitive fluorescent dye. In human umbilical vein endothelial cells, histamine triggers IP₃ accumulation and increases cytosolic Ca²⁺ that can be reversed by inhibition of protein kinase C (17).

We found that treating HeLa cells with histamine induced a transient increase in the concentration of cytosolic Ca²⁺ and that overexpression of NrzCb5 but not NrzBH4Cb5 partially inhibited this effect (Fig. 1, C and D), suggesting that the BH4 domain is not sufficient to inhibit IICR.

Nrz contains a total of four Bcl-2 homology (BH) domains numbered BH4, BH3, BH1, and BH2 from the N- to C-terminus (Fig. 1A). Thus, we asked whether longer N-terminal fragments of Nrz containing other BH domains could affect IICR. Consistent with the fact that BH4 domain of Nrz mediates its interaction with IP3R1, we found that all forms of C-terminally truncated Nrz co-immunoprecipitated with endogenous IP₃R1 (Fig. 1E). However, overexpression of the Nrz BH 1, 3 and 4 domains (Nrz1-94Cb5) significantly reduced histamine-induced increased cytosolic Ca²⁺, whereas overexpression of the Nrz BH 3 and 4 domains (Nrz1-67Cb5) did not (Fig. 1, F and G), suggesting that the BH1 domain is required for IICR.

Nrz inhibition of IICR is independent of its interaction with the pro-apoptotic protein Bax.

The fact that the BH1 domain of Nrz was required for its interaction with IP₃R1 could mean that the ability of Nrz to inhibit IICR is related to its ability to bind pro-apoptotic members of the Bcl-2 family. The BH1 domain of Bcl-2 family proteins contains a conserved Asn-Try-Gly-Arg motif. Mutation of Gly¹⁴⁵ to Ala in Bcl-2 prevents its heterodimerization with the pro-apoptotic protein Bax (18). To test whether the analogous residue in Nrz mediated dimerization with Bax, we mutated Gly⁸⁵ to Ala in otherwise wild-type Nrz (NrzG85A). We found that full-length wild-type Nrz but not NrzG85A or Nrz1-94 co-immunoprecipitated endogenous Bax (Fig. 2A) and reduced the abundance of cleaved poly ADP-ribose polymerase (PARP) induced by overexpression of Bax in HeLa cells (Fig. 2B), suggesting that the Gly⁸⁵ residue in the BH1 domain is important for Nrz-Bax-mediated apoptosis. Thus, we asked whether Gly⁸⁵ was required for Nrz to inhibit IICR. We fused the NrzG85A to the ER-targeting Cb5 domain (NrzG85ACb5) and expressed it in HeLa cells. We found that NrzG85ACb5 significantly reduced histamine-induced increases in cytosolic Ca²⁺ (Fig. 2C and 2D). Collectively, these results suggest that the function of Nrz to inhibit IICR is most likely independent of binding to Bax.

Nrz binds to the IP₃-binding domain of zIP₃R1

The BH4 domain of several anti-apoptotic Bcl-2 family proteins, including Bcl-2 and Bcl-xL, interact with either the MTD or CD of IP₃R1, which is determined by the primary sequence of the BH4 domains (5, 6, 16, 19-20). The primary sequence of the Nrz BH4 domain differs considerably from those of zBcl-2 and zBcl-xL (Fig. S1). To determine which domain of Nrz interacts with IP₃R1 we expressed fragments of zebrafish IP₃R1 (zIP₃R1) (Fig. 3A) in HeLa cells.

We found that the IP₃BD but not the SD, MTDs, or CD of zIP_3R1 co-immunoprecipitated with NrzCb5 (Fig. 3B) but not NrzCb5 lacking the BH4 domain (Nrz Δ BH4Cb5) (fig. S2), suggesting that the interaction between Nrz and IP₃R1 is mediated by the specific binding of the IP₃BD of IP₃R1 to the BH4 domain of Nrz.

Nrz binds outside the IP₃ binding pocket of IP₃R1

A group of residues in IP₃BD forms a positively charged pocket in the IP₃-binding site (24). In mouse IP₃R1, point mutations in these residues abolish the binding of both IP₃ (24) and IRBIT (IP₃R binding protein released with IP₃) a competitive inhibitor of IP₃ (25, 26). These residues are conserved in zebrafish; therefore, we asked whether they were required for binding to Nrz. Individual mutations to each of the corresponding amino acids did not affect the ability of the IP₃BD of zIP₃R1 to co-immunoprecipitate NrzCb5 (Fig. 3C) when co-expressed in HeLa cells, suggesting that, unlike IRBIT, Nrz does not act as a competitive inhibitor of IP₃ binding to IP₃R1.

To identify which residues could govern the interaction of IP₃R1 and Nrz, we performed molecular docking simulation between the IP₃BD of mouse IP₃R1 (PDB: 1N4K) and the BH4 domain of Nrz (Fig. 3D). This analysis suggested that Nrz interacts with IP₃R1 outside of the IP₃ binding pocket, consistent with the mutational analysis (Fig. 3C), and identified three residues in IP₃R1 that could mediate its interaction with Nrz. We mutated the corresponding residues in zIP₃R1 (Glu²⁵⁵, Glu⁴¹⁰, and Tyr⁵⁷⁶) and performed co-immunoprecipitation experiments in HeLa cells. We found that E225A prevented the interaction of NrzCb5 with the IP₃BD (Fig. 3E), indicating that Glu²⁵⁵ of zIP₃R1 was critical for binding to Nrz. Analysis of docking solutions also indicated that the interaction of Nrz and IP₃R1 may depend on the interaction between zIP₃R1 Glu²⁵⁵ and Cys²⁰ of Nrz. Analysis of primary sequence alignments demonstrated that Cys²⁰ is highly conserved across Nrz orthologs (Fig. S3), suggesting that this residue could be important for binding between Nrz and zIP₃R1 and that this interaction could be evolutionary conserved.

Nrz prevents IP₃ from binding to IP₃R1

We found that Nrz inhibits IICR and binds to the IP₃BD of IP₃R1, suggesting that it could interfere with IP₃ binding to IP₃R1. We verified that Nrz does not affect the steady state concentration of Ca^{2+} in the ER. In HeLa cells, expression of NrzCb5 did not affect increased cytosolic Ca^{2+} caused by treatment with thapsigargin (Fig. S4), which inhibits the sarco-endoplasmic reticulum Ca^{2+} ATPase and thereby flushes Ca^{2+} from the ER (21).

To test whether Nrz could prevent binding of IP₃ to IP₃R1 to inhibit IICR, we used the IP₃R-based IP₃ sensor (IRIS), which contains the IP₃BD flanked by enhanced cyan fluorescent protein (ECFP) and the yellow fluorescent protein Venus. When IP₃ binds to IRIS, it induces a conformational change in the IP₃BD that decreases fluorescent resonance energy transfer (FRET) from ECFP to Venus (22).

Treating HeLa cells expressing IRIS with histamine induced a rapid increase in the ratio (R) between ECFP and Venus fluorescence intensities indicating reduced FRET (Fig. 4A). Transient overexpression of full-length wild-type Nrz or Nrz1-94, but not NrzBH4 nor Nrz Δ BH4, prevented histamine-induced change in IRIS FRET ratio (Fig. 4, A and B). We also directly assessed the binding of IP₃ to IP₃R1 using fluorescence polarization (23) to measure the interaction between IP₃ covalently bound to fluorescein isothiocyanate (IP₃-FITC) and a recombinant protein comprised of the SD and IP₃BD of IP₃R1 (IP₃R1-NTD). When we incubated IP₃-FITC with IP₃R1-NTD in the presence of recombinant Nrz, we found that Nrz reduced fluorescence polarization in a concentration-dependent manner (half maximal inhibitory concentration = 2.96 x 10⁻⁸ ± 1.77 x 10⁻⁸ M) (Fig. 3C). Collectively, these results demonstrate that Nrz inhibits the binding of IP₃ to IP₃R1.

Phosphorylation of Nrz inhibits its interaction with IP₃R1

We hypothesized that the interaction between endogenous Nrz and IP₃R1 could be regulated by signal transduction mechanisms such as phosphorylation. Phosphorylation of Bcl-2 on a Thr and two Ser residues in the loop between its BH4 and BH3 domains increases Ca²⁺ release from the ER (27). Similar to Bcl-2, Nrz has a Thr and two Ser residues in the loop between the BH4 and BH3 domains (Fig. 5A) that could be phosphorylated. We mutated these three residues to generate phosphomimetic (T26D, S29D, S31D, NrzDDD) and non-phosphorylatable (T26A, S29A, S31A, NrzAAA) amino acid versions of Nrz. When transiently expressed in HeLa cells, NrzAAACb5 but not NrzDDDCb5 significantly decreased histamine-induced increased cytosolic Ca²⁺ (Fig. 5, B and C). Moreover, NrzAAACb5, but not NrzDDDCb5, co-immunoprecipitated the IP₃BD of IP₃R1 (Fig. 5D). To identify which of these three amino acids was important for Nrz interation with IP₃R1, we generated single and double mutants at each position. We found that Nrz mutants containing S31D did not co-immunoprecipitate the IP₃BD of IP₃R1 (Fig. 5E), suggesting that Ser³¹ phosphorylation by an unknown kinase could play a critical role in inhibiting the interaction between Nrz and IP3R1 and promoting IICR.

Domains of Nrz required for the inhibition of IICR are also required for zebrafish epiboly

We previously reported that Nrz knockdown by antisense morpholino (MO) injection results in the constriction of the blastoderm margin and the detachment of the embryo from the yolk cell during epiboly (13). The injection of *nrz* MO induces a robust increase in cytosolic Ca²⁺ and embryonic lethality that can be prevented by co-injecting mRNA encoding NrzCb5 but not Nrz Δ BH4Cb5 (14). Here, we found that both the BH4 and BH1 domains of Nrz were required to inhibit IICR in HeLa cells (Fig. 1). Therefore, we tested whether these domains were sufficient to reverse the phenotype induced by *nrz* MO injection.

We co-injected embryos with *nrz* MO and in vitro synthesized mRNA encoding various truncated forms of Nrz and monitored the percentage of embryos showing abnormal margin constriction during epiboly (Fig. 6A). Co-injection of *nrzbh4cb5* did not prevent epiboly defects induced by *nrz* MO (Fig. 6, A and B), consistent with the observation that the BH4 domain was not sufficient to reduce IICR in HeLa cells. Moreover, co-injection of *nrz1-94cb5* but not *nrz1-67cb5* also prevented epiboly defects induced by *nrz* MO (Fig. 6C), demonstrating a requirement for the BH1 domain of Nrz in this process. We also found that the ability of Nrz to prevent IICR was independent of its ability to bind to Bax in HeLa cells (Fig. 2C). Similarily, co-injection of *nrzG85A*, which encodes a form of Nrz that does not bind Bax (Fig. 2A), prevented epiboly defects induced by *nrz* MO (Fig. 6D). Finally, we found that Nrz phosphorylation could impair its interaction with IP₃R1 in HeLa cells (Fig. 5D). In zebrafish, co-injection of *nrzDDDcb5* reduced the percent of embryos with epiboly defects induced by *nrz* MO (Fig. 6E). Thus, the domains and residues of Nrz required to inhibit IICR in cultured cells are also required for zebrafish epiboly.

Phosphorylation of Nrz enables Ca²⁺ signaling, actin-mysoin ring formation, and epiboly

During epiboly, IP₃-dependent cyclic increases in cytosolic Ca²⁺ concentration, referred to as Ca²⁺ waves, occur in the YSL (10), where *nrz* is expressed (13). Because Nrz inhibits IP₃ binding to IP₃R1 in HeLa cells (Fig. 4, A-C), we hypothesized that Nrz activity is negatively regulated to enable the generation of Ca²⁺ waves. In HeLa cells, we found that mutants of Nrz that mimic phosphorylated Thr and Ser do not bind to IP₃R1 (Fig. 5D) or inhibit IICR (Fig. 5C). To examine whether Nrz is phosphorylated during epiboly in vivo, we injected *nrzcb5* mRNA and immunoprecipitated NrzCb5 from ER fractions prepared from the YSL of embryos at different developmental stages. Western blot analysis revealed that Nrz Ser phosphorylation was barely detectable before the onset of epiboly and increased robustly by 30% epiboly (Fig. 7A), suggesting that Nrz phosphorylation could be important to enable IP₃-dependent Ca²⁺ signaling in vivo during this process.

We tested whether replacing endogenous Nrz with a non-phosphorylatable form of Nrz could disrupt Ca^{2+} signaling. In wild-type embryos injected at the 128-cells stage with a Ca^{2+} sensitive dye, we observed cyclic Ca^{2+} waves in the YSL at 30% epiboly (Fig. 7, B-E; Movie S1). To evaluate the contribution of Nrz phosphorylation to the generation of these Ca^{2+} waves, we co-injected *nrz* MO and *nrzAAAcb5* or *nrzcb5*. We found that *nrzcb5* but not *nrzAAAcb5* restored normal Ca^{2+} waves in the absence of endogenous Nrz, (Fig. 7, B-E; Movies S2 and S3), suggesting that the phosphorylation of Nrz enables the generation of YSL Ca^{2+} waves at the beginning of epiboly.

An actin-myosin ring forms at the blastoderm margin in zebrafish embryos at 40% epiboly, and localized disruption of actin in the YSL at 60% epiboly delays cell movements associated with epiboly progression (12).

Similarly, treating zebrafish embryos at 50% epiboly with cytochalasin B, which inhibits actin network formation, or with a Ca²⁺ chelator disrupts actin ring formation and epiboly (28), suggesting that Ca²⁺ waves are important for the formation of the actin-myosin ring. Given that *nrzAAAcb5* disrupted Ca²⁺ waves when co-injected with *nrz* MO, we examined the formation of the actin-myosin ring. Co-injection of *nrz* MO with *nrzAAAcb5* but not *nrzcb5* nor *nrz* MO injection alone reduced F-actin staining at the blastoderm margin at 50% epiboly (Fig. 8, A and B), suggesting that the expression of non-phosphorylatable Nrz inhibits YSL Ca²⁺ dynamics and thereby disrupts the formation of the actin-myosin ring. The above data suggested that the phosphorylation of *Nrz* could be important for epiboly progression. In embryos injected with *nrz* MO, co-injection of *nrzAAAcb5* but not *nrzcb5* resulted in epiboly delay (Fig. 8C) or arrest (Fig. 8D). Collectively, these results suggest that by 50% epiboly endogenous Nrz is mostly phosphorylated and cannot bind to IP₃R1 and is thus permissive to Ca²⁺ signaling required for actin assembly and contractility.

Discussion

We found that Nrz binds outside the IP₃ binding site on the IP₃BD of IP₃R1 and prevents IP₃ binding, revealing a new regulatory mechanism for IP₃Rs by Bcl-2 related proteins. Bcl-2 reduces Ca²⁺ release by interacting with the MTD of IP₃R1 (5, 15), and Bcl-xL sensitizes IP₃R1 to low IP₃ concentrations by interacting with the CD (6). The differential binding of Bcl-2 proteins to various domains of IP₃R1 may be determined by divergent amino acid sequences in the BH4 domains (16, 20).

Here, we found that the BH4 domain of Nrz was sufficient for it to interact with IP₃R1, suggesting that residues within this domain could be critical for determining the interaction with IP₃BD. Molecular docking simulation identified a conserved Cys (Cys²⁰ in Nrz) as a potential factor in mitigating the binding of Nrz to the IP₃BD of IP₃R1. We found Glu²⁵⁵ of zIP₃R1 most likely contacts Nrz Cys²⁰ and is essential for binding between these proteins. Whether Nrz Cys²⁰ is required to bind IP₃R1 and whether analogous residues confer specificity of binding of Bcl-2-family and other proteins to IP₃BDs remains to be determined.

Several proteins interact with the IP₃BD or decrease IP₃ binding to the IP₃R1. Beclin-1 binds to the IP₃BD of IP₃R1, but Beclin-1 knockdown has no effect on Ca²⁺ homeostasis (30). Sodium-potassium adenosine triphosphatase binds to the IP₃BD of IP₃R1 and promotes Ca²⁺ release in the absence IP₃ (31). Carbonic anhydrase-related protein (CARP) inhibits IP₃ binding to IP₃R1 by reducing the affinity of the receptor for IP₃ (32). CARP interacts with the MTD of IP₃R1 (32), suggesting that Nrz and CARP act by distinct mechanisms.

IRBIT binds the IP₃BD of IP₃R1 and directly competes with IP₃ for binding to its receptor (25, 26). We found that mutations in residues of IP₃R1 required for interaction with IRBIT and IP₃ were not required for binding to Nrz, suggesting that Nrz and IRBIT act by distinct mechanisms to inhibit IP₃R1.

The Nrz BH4 domain binds outside the IP₃ binding site of IP₃R1, and the inhibition IICR by Nrz requires the N-terminal most 94 amino acids including the BH4, BH3 and BH1 domains. The BH4 and BH3 domains combined were not sufficient to inhibit IICR without the BH1 domain, despite still being able to bind to IP₃R1. These findings imply that Nrz is an allosteric inhibitor. Binding of Nrz could produce a conformational change of the IP₃BD of IP₃R1 that decreases the affinity of the IP₃ binding site for IP₃. Alternatively, Nrz, potentially involving its BH1 domain, could hinder access of IP₃ to the IP₃ binding site on IP₃R1 without direct competition for key residues.

The mutation of putative phosphorylated residues to phospho-mimetic amino acids in Nrz prevented its ability to inhibit IICR and bind IP₃R1. Mutation of analogous residues in Bcl-2 to non-phosphorylatable amino acids enhances its ability to reduce Ca^{2+} release from the ER (27). However, this affect is indirect. Phosphorylation of Bcl-2 reduces its interactions with pro-apoptotic proteins, such as Bax, which increases ER Ca^{2+} content by reducing the Ca^{2+} leak (33). Nrz did not alter basal ER Ca^{2+} concentration and inhibition of IICR by Nrz did not require interaction with Bax. Moreover, phospho-mimetic mutations in Nrz prevented interaction with the IP₃BD of IP₃R1, suggesting that Nrz acts directly on IP₃R1-dependent Ca^{2+} release in a manner that depends on Nrz phosphorylation.

We found that Nrz was phosphorylated during early zebrafish epiboly, a process that requires Ca^{2+} signaling (28), and expression of Nrz with non-phosphorylatable amino acids suppressed cyclic Ca^{2+} waves at the beginning of epiboly, compromised actin-myosin ring formation at the blastoderm margin, and delayed or disrupted epiboly. Ca^{2+} waves begin at the time when the actin-myosin ring is formed (12, 28) and our data are consistent with the fact that Nrz must be inhibited to enable this process. We observed that the period of Ca^{2+} waves in the YSL was approximately seven minutes.

During myofibrillogenesis, actin-myosin network assembly is regulated by Ca^{2+} transients with a similar period (34, 35). At the blastoderm margin, actin associates with phospho-myosin-II (36). We previously demonstrated that increased cytosolic Ca^{2+} concentrations in embryos injected with *nrz* MO promotes myosin light chain (MLC) phosphorylation, which leads to premature actin-myosin ring formation at 30% epiboly (14). Thus, Ca^{2+} waves during early epiboly could time MLC phosphorylation and the progressive assembly of the actin-myosin ring.

Our results identify the role of Nrz in IICR and emphasize the role of Bcl-2 family proteins in the regulation of intracellular Ca^{2+} . We recently demonstrated that Bcl-wav, a newly characterized Bcl-2 family member, is essential for convergence and extension movement in zebrafish gastrulation. During this process, Bcl-wav regulates the formation of actin protrusions via its role in mitochondrial Ca^{2+} uptake (38). Taken together, these studies emphasize the diversity of functional roles of Bcl-2 family proteins in numerous cellular processes.

Materials and Methods

MOs, reagents and antibodies

The *nrz* MO was designed according to the manufacturer's recommendations (Gene Tools, LLC) and had the following sequence: 5' CATTTTCCTCCCAGCGATGTCAGAC 3'. A second MO that contained 4 mismatches relative to *nrz* MO was used as a negative control (controlMO). The sequence was as follows: 5' CATT<u>A</u>TCCT<u>G</u>CCAGC<u>C</u>ATGT<u>G</u>AGAC 3'(13).

The following antibodies were used: IP₃R1 (PA1-901, Thermo Scientific), rabbit FLAG (F-7425, Sigma-Aldrich), mouse FLAG (F-3165, Sigma-Aldrich), rabbit HA (Ab75640, Abcam), mouse HA (MMS-101P, Covance), PARP (Ab6079, Abcam), Vinculin (SC-55465, Santa Cruz Biotechnology), Bax (5023, Cell Signaling), phosphoserine (05-1000, Millipore).

Vector constructions and mRNA in vitro transcription

Nrz and *zbax* open reading frames were cloned into the pCS2+ expression vector as previously described (13). The transmembrane domain of Cb5 was cloned in pCS2+ as previously described (14). Mutants of Nrz were inserted between the ClaI and XhoI restriction sites in the pCS2+Cb5 or pCS2+ vectors. For cloning of *zip₃r1*, total RNA from embryos was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions, and complementary DNA was generated with a cDNA synthesis kit (iScript cDNA synthesis kit, Biorad) using 500 ng of total RNA and gene specific primers. An HA-tag was added to the N-terminus by PCR. Gene fragments corresponding to the IP₃R1 domains were amplified and cloned between ClaI and XhoI or EcoRI and XhoI sites in pCS2+. pCDNA3.1-IRIS was kindly provided by Pr. Mikoshiba (22). In vitro transcription for the different constructs was performed using the SP6 mMESSAGE mMACHINE kit according to the manufacturer's recommendations (Ambion).

Zebrafish husbandry, manipulation, injection and analysis

Zebrafish (crosses between AB and TU or AB and TL strains) were raised and maintained according to the standard procedures (39). Zebrafish embryos were grown in E3 Medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, pH 7.6) at 28.5°C.

Morpholino (0.5 mM) and mRNA (100 ng/ml) injections were performed between the one- and fourcell stages. Embryos were then observed every 15 min and embryos showing margin constriction and cell detachment from the yolk were recorded as "embryos with a phenotype". Calcium Green-1 Dextran (Invitrogen) at 250 μ M was injected at the 128-cell stage into the top of the yolk, close to the margin of the yolk and blastoderm. Images of embryos at 8 hpf were acquired using a *Nikon TE30* inverted microscope and Metamorph Software. Epiboly was quantified using Image J software by measuring the distance of the blastoderm margin from the animal pole (D) and the size of the embryos (S). Epiboly progression data were obtained by using the ratio D/S. F-Actin staining of embryos was performed as previously described (35). In brief, embryos at the desired stage were fixed overnight in 4% paraformaldehyde at 4°C and washed in 0.1% Triton in PBS (PBT). They were then permeabilized for 1 h in 0.5% Triton in PBS and subsequently incubated in block solution (10% goat serum, 1% DMSO, 0.1% Triton in PBS) for 5 h. Embryos were then incubated in Rhodamine-Phalloidin (Invitrogen) overnight at 4°C and washed three times in PBT.

Embryos use for Ca^{2+} imaging were injected with Calcium Green-1 Dextran (Invitrogen) at 250 μ M into the top of the yolk at blastoderm margin of 128-cell stage embryos (10), dechorionated, and mounted in low melting point 0.7% agarose in E3 medium on the stage of a Zeiss LSM 780 microscope and incubated at 28.5°C. Stacks of 4 μ m were acquired and the sum of the first three stacks was utilized to visualize variations in Ca²⁺ concentration in the external YSL.

ER from the YSL was isolated as described previously (14). The 100,000 x g pellet was re-suspended in TNE buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA (pH 7.4), 1 mM β glycerophosphate, 1 mM orthovanadate, 0.1 mM sodium pyrophosphate containing protease inhibitors) and was used for FLAG immunoprecipitation.

Cell culture, immunoprecipitation, Ca²⁺imaging, and FRET

HeLa cells were grown in DMEM high glucose (Gibco) supplemented with 10% FBS and 1% Penicillin-Streptomycin (Gibco) at 37°C in 5% CO₂ humidified atmosphere.

For endogenous IP₃R and Bax co-immunoprecipitation experiments, $6x10^6$ HeLa cells were transfected with the indicated vectors. After 24 h, cells were lysed in TNE buffer. Extracts were precleared with protein G-Sepharose beads for 1 h at 4°C and then incubated overnight with 5 µg of IP₃R or Bax primary antibody. The extracts were then incubated with protein G-Sepharose beads for 2 h. Immunoprecipitated fractions were washed three times with TNE and analyzed by immunoblotting. For HA and FLAG immunoprecipitation, $1x10^6$ HeLa cells were transfected with the indicated vectors and lysed and processed as above. 1 µg of primary rabbit HA or rabbit FLAG was used.

For Ca²⁺ imaging, HeLa cells cultured in NuncTM LabTek® chambered coverglass were incubated with 5 μ M FluoForte (Enzo Life Sciences) in a Ca²⁺-free Balanced Salt Solution (BSS) (121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 1.8 mM CaCl₂, 6 mM NaHCO₃, 5.5 mM D-glucose, 25 mM HEPES, pH 7.3) for 1 h at 37°C. Fluorescence values were collected using a Zeiss LSM 780 confocal microscope. After 10 s of measurement, 5 μ M histamine (Sigma) or thapsigargin (Enzo Life Sciences) in Ca²⁺-free BSS was injected.

For IRIS FRET, HeLa cells cultured in NuncTM LabTek® chambered coverglass were co-transfected with pcDNA3.1-IRIS (22) and pCS2+ encoding the various Nrz mutants (n_{mole} ratio = 1:3). Before imaging, the medium was replaced with BSS. FRET was measured with a Zeiss LSM 780 confocal microscope by excitation at 405 nm. ECFP and Venus fluorescence were detected at 450-510 nm and 525-565 nm, respectively. After 10 s of measurement, 5 μ M histamine in BSS was injected. Changes in the FRET signal were calculated using the F_{ECFP}/F_{Venus} ratio (R).

*IP*₃-*FITC fluorescence polarization measurement.*

IP₃-FITC, IP₃ and the recombinant N-terminal domain protein were purchased in the Hit Hunter Inositol (1,4,5)-Trisphosphate Assay kit from DiscoveRx (Fremont, CA). Recombinant Nrz protein was produced as previously described (13). IP₃-FITC at 0.5 nM was incubated with 4 nM of recombinant NTD and the various recombinant Nrz proteins for 30 min at 25°C. Fluorescence polarization was recorded at 25°C with 485 nm excitation and 530 nm emission filters using a Mithras LB 940 multimode microplate reader (Berthold Technologies).

Sequence alignment

Nrz ortholog sequences were found in NCBI protein database. Sequence alignment was performed using the Clustal Omega tool at <u>http://www.ebi.ac.uk/Tools/msa/clustalo/.</u> An Image of the alignment was obtained using the Jalview software (40).

Molecular docking simulation

The Nrz protein structure was obtained by analyzing the Nrz amino-acid sequence on the Phyre² (41) homology modeling server. The Nrz BH4 domain structure was extracted and a docking experiment was performed against the IP₃R1 Binding Domain crystal structure (pdb: 1N4K) using the PatchDock server (42). Images were acquired using UCSF Chimera software (43).

Statistical Analysis

Statistical significance was analyzed using the Mann-Whitney U-test; p < 0.005 was considered significant.
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Author contributions: BB, AN, JP and NP performed the experiments. BB, NP, RR and GG designed the experiments. BB and GG wrote the manuscript. **Competing interests:** The authors declare that they have no competing interests.





A, Schematic of full-length Nrz and the Cb5 domain of cytochrome B. Lines and colored boxes represent α -helices and BH domains, respectively. **B**, Representative Western blot of immunoprecipitation (IP) between endogenous IP₃R1 and FLAG-NrzBH4 or FLAG-NrzCB5. N=3. **C**, Representative Ca²⁺ response curve of cells stimulated with 5 μ M histamine at the indicated time. Cells were transfected with the empty vector, FLAG-NrzBH4Cb5, or FLAG-NrzCb5. **D**, Histogram showing the mean amplitude (±SD) of the histamine-induced Ca²⁺ peak.

N≥5 fields from biological replicates. **E**, Representative Western blot of IP between endogenous IP₃R1 and FLAG-tagged C-terminal Nrz deletion mutants. N=3. **F**, Representative Ca²⁺ response curve of cells stimulated with 5 μ M histamine at the indicated time. Cells were transfected with the empty vector, FLAG-Nrz1-67Cb5, or FLAG-Nrz1-94Cb5. **G**, Histogram showing the mean amplitude (±SD) of the histamine-induced Ca²⁺ peak. N≥5 fields from biological replicates. *, p < 0.005 (Mann-Whitney U-test) for **D** and **G**.



Fig. 2. Nrz regulates IICR in HeLa cells independent of its interaction with Bax.

A, Representative Western blot of IP between endogenous Bax and FLAG-tagged Nrz, Nrz1-94 or NrzG85A. N=3. **B**, Representative Western blot cleaved-PARP (c-PARP) in cells transiently expressing zBax (zebrafish Bax) and FLAGtagged full-length Nrz, Nrz1-94 or NrzG85A. N=3. **C**, Representative Ca²⁺ response curve of cells stimulated with 5 μ M histamine at the indicated time. Cells were transfected with the empty vector or FLAG-NrzG85ACb5. **D**, Histogram showing mean amplitude (±SD) of the histamine-induced Ca²⁺ peak. N≥5 fields from biological replicates. *, p < 0.005 (Mann-Whitney U-test).



Fig. 3. Nrz binds the IP₃BD of zIP₃R1 in HeLa cells.

A, Schematic of zIP₃R1 domains. **B**, Representative Western blot of IP between HA-tagged zIP₃R1 domains and FLAG-NrzCb5. Bands corresponding to the zIP₃R1 domains were indicated with a black arrow. N=3. **C**, Representative Western blot of IP between HA-tagged IP₃BD or IP₃BD binding site mutants and FLAG-NrzCb5. N=3. **D**, Docking model of the BH4 domain of Nrz (modeled by multi-template homology with Phyre²) binding to the IP₃BD of mouse IP₃R1 (PDB: 1n4k). The red dashed circle represents the IP₃ binding site. **E**, Representative Western blot of IP between HA-tagged IP₃BD domain or IP₃BD mutants (E255A, E410A, Y576F) and FLAG-NrzCb5. N=3.



Fig. 4 Nrz prevents IP₃ binding to IP₃R1.

A, Representative curve of the FRET signal change of IRIS in HeLa cells stimulated with 5 μ M histamine and expressing the indicated proteins. The FRET signal was calculated as the ratio (R) of fluorescence emission of ECFP to the fluorescence emission of Venus with excitation at 405 nm. **B**, Histogram showing the mean FRET signal change (±SD) induced by histamine stimulation. N=30 cells from at least 3 biological replicates. **, p < 0.0001 (Mann-Whitney U-test); n.s, non-significant, p > 0.1. **C**, Curve showing the mean fluorescence polarization (± SD) of IP₃-FITC in the presence of IP₃R1 NTD and the indicated concentration of recombinant Nrz protein. N=3 independent triplicate measurements.



Fig. 5. Phospho-mimetic mutant Nrz does not bind IP₃R1 or inhibit IICR in Hela cells.

A, Primary sequence of the Nrz protein including the BH4 and BH3 domains. Putative phospho-serines and phosphothreonine are shown in red. **B**, Representative Ca²⁺ response curve of cells stimulated with 5 μ M histamine at the indicated time. Cells were transfected with the empty vector, FLAG-NrzAAACb5, or FLAG-NrzDDDCb5. **C**, Histogram showing the mean amplitude (±SD) of the histamine-induced Ca²⁺ peak. N≥5 fields from biological replicates. *, p < 0.005 (Mann-Whitney U-test). **D**, Representative Western blot of IP between HA-tagged IP₃BD and FLAG-tagged NrzAAACb5 or NrzDDDCb5. N=3. **E**, Representative Western blot of IP between HA-tagged IP₃BD and FLAG-tagged Nrz or phosphomimetic Nrz mutants.



Fig. 6. Nrz requires its BH4-BH1 region to regulate zebrafish epiboly.

A, Representative bright field images at 50% epiboly of the phenotype (constriction at the blastoderm margin) induced by the injection of *nrz* MO alone or in combination with *nrzcb5* or *nrzbh4cb5* mRNA. Scale bar = 100 μ m. (B-E) Histograms showing the mean percentage (±SD) of embryos displaying the phenotype in (A) at 8 h post-injection (hpi). N≥3 independent injections (80-100 embryos per injection per condition). *, p < 0.005 (Mann-Whitney U-test).



Fig. 7. Nrz phosphorylation is required for generation of cyclic Ca²⁺ waves in the YSL.

A, Representative Western blot of IP for FLAG-NrzCb5 from ER of YSL cells isolated from embryos at the indicated stages N=3. **B**, Ca²⁺ concentration ([Ca²⁺]) in the external-YSL of uninjected wild-type (WT) embryos or embryos injected with *nrz* MO and *nrzcb5* or *nrzAAAcb5* mRNAs. Scale bar = 20 μ m. **C**, Representative graph of [Ca²⁺] variation shown in (B). (**D** and **E**) Histograms showing the amplitude (D) or period (E) (mean ± SD) of YSL [Ca²⁺] as in (C). *, p < 0.005 (Mann-Whitney U-test). N≥6 embryos per condition.



Fig. 8. Nrz phosphorylation is required for epiboly progression and actin ring formation.

A, Phalloidin-rhodamine staining of F-actin in embryos at the shield stage injected with *nrz* MO alone or in combination with *nrzcb5* or *nrzAAAcb5* mRNA. White arrows indicate the actin-myosin ring. **B**, Histogram showing the relative actin fluorescence (mean±SD). Relative fluorescence is indicative of the amount of F-actin at the blastoderm margin and was calculated by using the ratio of the rhodamine fluorescence at the blastoderm margin to the rhodamine fluorescence in the blastomeres. N = 30 embryos per condition. **C**, Histogram showing the mean percentage (±SD) of epiboly progression at 8 h post-fertilization (hpf). N=90 embryos from 3 independent injections. **D**, Representative images of the phenotypes (epiboly arrest) of embryos at 5 hpf injected with *nrz* MO and *nrzcb5* or *nrzAAAcb5* mRNA. Nrzcb5 restores epiboly progression (50% epiboly) in contrast to NrzAAAcb5 (epiboly arrest). N=3 independent injections (80-100 embryos per injection and condition). Scale bar = 20 μ m. *, p < 0.005 (Mann-Whitney U-test) for **B-D**.

Supplementary figures



Fig. S1: Sequence alignment of BH4 domains of Nrz, zBcl-2 and zBcl-xL.

The BH4 domain of Nrz, zBcl-2 and zBcl-xL were aligned using Clustal Omega tool. Residues conserved between domains were highlighted in blue. Identical residues in the three domains were surrounded in red.



Fig. S2: Nrz interaction with IP₃BD requires its BH4 domain.

Lysates from HeLa cells transiently expressing the indicated proteins were subjected to FLAG IP. Interaction was detected by western blotting with HA antibody. N=3 blots

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Danio rerio (NP_919379)	MS	C	-	-	-	W	L/L	R	Е	Q	т	LI	L	LA	١	E (2	1	S	F	С	s	G	1	Q	ן ב	P	Ρ	s	Е
Salmo salar (ACI32987)	MS	C	-	-	-	G	L	w	К	E	т	L	A	1.	A	EC	`	L	s	v	С	s	I	L	Ρ.	AS	G	S	w	к
Gadus morhua (ACZ62638)	MS	C	-	-	-	R	L	W	К	E	Т	L	A	L	5	EC	2	/ L	L	s	С	т	A	s	Т	G 1	A	Ρ	Ρ	Ρ
Osmerus mordax (ACO09739)	MS	C		-	-	R	L	W	К	E	Т	L.	A	LO	5	EC	2	1 L	A	F	С	с	A	s	Ρ	RL	A	P	Ρ	Ρ
Xenopus tropicalis (XP_002936386)	МΤ	C) -	-	-	Q	L	к	Е	E	Г	c	A	LI	L	GE	1	r L	Q	н	С	A	s	Р	Q	DE	K	A	L	s
Homo sapiens (NP_065129)	MA	C) -	-	-	Ρ	L	R	Е	R	Т	Е	L	LL	. /	1	٦Y	Ĺ	G	Y	С	A	R	Е	P	GΤ	P	Е	Ρ	A
Pan troglodytes (XP_523079)	MA	C) -	-	-	Ρ	L	R	Е	R	Т	E	R	LI	L	A	٦Y	Ĺ	G	Y	С	A	R	Е	P	GΤ	P	Е	Ρ	т
Nomascus leucogenys (XP_003266987)	ΜV	C) -	-	-	Q	L	R	Е	R	Т	E	R	LI	L /	A I	٦Y	Ľ	G	с	С	A	R	Е	P	G 1	P	Е	Ρ	т
Mus musculus (NP_038507)	MA	C	5	Q	D	P	L	Н	Е	R	Т	R	R	LI		5 1	٥١	'	F	F	с	A	R	Е	Ρ	DT	-	-	-	Ρ
Rattus norvegicus (NP_446185)	MG	D) -	-	-	Ρ	L	Q	D	R	Т	R	R	LI		Г	DY	1	L	F	С	A	R	А	Ρ	NT	P	Е	Ρ	L
Felis catus (XP_003987226)	MA	C) -	-	-	A	L	R	Е	R	Т	A	Q	LI	L '	Г	٦Y	L	Е	Y	С	A	R	Е	P	G S	P	Α	R	т
Bos taurus (XP_601350)	ΜV	C) -	-	-	Ρ	F	R	Е	R	т	A	R	LI	L	M	2	/ L	Е	F	С	A	R	Е	P	G 1	P	A	Ρ	A
Ovis aries (XP_004011065)	ΜV	C) -	-	-	Ρ	F	R	Е	R	т	A	R	LI	LI	M) V	۷L	Е	F	С	А	R	Е	P	G 1	P	Α	Ρ	Α
Ceratotherium sinum (XP_004422061)	MA	C) -	-	-	A	L	R	Е	R	Т	A	R	LI	L 1	Г	00	L	Е	Y	С	A	R	Е	P	GΤ	A	A	G	A
Odobenus rosmarus (XP_004396639)	MA	C) -	-	-	A	L	К	Е	R	т	т	R	LI		Г	DY	Ĺ	Е	Y	С	A	R	Е	P	GТ	P	Α	R	A
Coturnix coturnix (CAA59136)	MP	G	-	-	-	S	L	К	Е	E	Г	A	L	LL	. 8	. 1	٥١	F	Q	н	R	A	G	G	A	A L	P	Ρ	s	Α
Gallus gallus (NP_989853)	MP	G	-	-	-	S	L	К	E	E	Г	A	L	LL	. 6	. 1	D Y	F	Q	н	R	A	G	G	A	A L	Ρ	Ρ	S	A

BH4 domain

Fig. S3. Sequence alignment of BH4 domains of Nrz orthologs.

BH4 domain containing sequences of Nrz orthologs from the indicated species were aligned using Clustal Omega tool. Conserved cysteines corresponding to Cys²⁰ of Nrz were surrounded with grey box and highlighted with black arrow. Accession number is indicated in brackets.



Fig. S4: Nrz do not reduce ER Ca²⁺ content.

A, Representative response curve of HeLa cells loaded with the calcium-sensitive dye FluoForte, transiently expressing NrzCb5 or transfected with empty vector and treated with 5 μ M thapsigargin at the indicated time. **B**, Histogram showing mean amplitude (± SD) of the thapsigargin-induced Ca²⁺ peak. N≥5 independent cell fields. **C**, Histogram showing mean area under curve (AUC) (±SD) of the thapsigargin-induced Ca²⁺ peak. Peak amplitude and AUC are indicative of ER Ca2+ content (21). N≥5 independent cell fields. n.s, non-significant, p > 0.3 (Mann-Whitney U-test) for **B** and **C**.

Discussion relative to Article 1

With this study in Science Signaling, we have shed a new light on the mechanism involved to allow Nrz suppressing of calcium signals. The domain involved in this interaction was showed to be the ligand binding domain of zIP3R1, unlike Bcl-2 or Bcl-xL that would respectively bind the human equivalent of zIP3R1 MTDII domain and CD (Monaco et al., 2012a; 2012b). Until this time, no interaction was reported between a Bcl-2 protein and the IP3 Binding Domain of IP3Rs. We sought to understand how Nrz was precisely affecting zIP3R1 calcium release, by mutating residues important for the IP3 fixation within IP3 binding pocket of zIP3R1 BD. Amazingly, mutations of these amino acids had no effect on Nrz binding, but a mutation within IP3 BD α domain (E255) was able to inhibit Nrz binding to IP3R1, thus reducing IP3 affinity in a dose-dependent manner. Together, these results demonstrate that Nrz is not a competitive inhibitor, but might rather act as an allosteric modulator of IP3 binding to IP3Rs.

Interestingly, it is worthwhile to mention that Nrz expressed in HeLa cells was able to interact with human IP3R1 and suppress calcium signaling. Nonetheless, the reverse interaction appeared not true, as the human counterpart of Nrz, BCL2L10/Nrh was unable to bind zebrafish IP3R1 binding domain (data not shown).

Nrz BH4 domain was showed to be required for the interaction with IP3R1, as its deletion prevent the interaction between dBH4 Nrz and IP3R1. Nevertheless, Nrz BH4 domain only or Nrz BH4-BH3 (Nrz 1-67) constructs were showed to be insufficient for suppression of calcium signals and IP3 BD conformational reorganization upon IP3 binding. A longer portion of Nrz protein was needed to regulate calcium fluxes, noted Nrz 1-94, comprising Nrz BH4, BH3 and part of BH1 domain.

As a consequence it can be speculated that Nrz inhibition of calcium signals is due to two different hypothesis: (1) Steric volume of BH3 and part of BH1 in the IP3 binding pocket behind Nrz BH4 interacting domain would prevent IP3 BD conformational rearrangements induced by ligand binding, hence decreasing the affinity for IP3 and efficiency of IP3-induced calcium release. (2) Interaction with an additional protein partner through the BH3 and part of BH1 domain, which can affect IP3R1 ability to release calcium ions.

Thus, we can further speculate that a peptide derived from Nrz BH4 may act as a decoy for full-length Nrz at its interaction site on IP3R1, but would not regulate calcium fluxes. This interesting strategy would work only for Nrz, but not Bcl-2, as it has been demonstrated that Bcl-2 BH4 domain only is sufficient to regulate negatively calcium signaling (Monaco et al., 2012a).

Phosphorylation of Nrz added yet another layer of regulation, as phosphomimetic mutant Nrz DDD (T26D-S29D-S31D) lost its activity to bind IP3 BD and suppress calcium signals. However, we showed that Nrz phosphorylation was required to ensure completion of gastrulation in zebrafish, as the actinmyosin ring formation is needed for this process.

Indeed, we observed that complementation of Nrz MO phenotype by Nrz AAA phosphonegative mutant (T26A-S29A-S31A) do not display an early actin-myosin ring, and its absence leads to a global epiboly delay, and sometimes arrest. As a consequence, it was supposed that Nrz suppression of calcium signals would be a transitory event, especially to ensure that the actin-myosin ring formation and contraction happen at the right moment and the right place.

Close control on Nrz phosphorylation would be the key signaling event governing gastrulation progression. In the present study, we failed to find an upstream kinase able to phosphorylate Nrz, partly because no kinase consensus site was found corresponding to T26/S29/S31 sites.

Candidates approach has also been unsuccessful, using inhibitors of some kinases known to control Bcl-2 calcium regulation capacity. A global proteomic analysis to identify kinases within the Yolk Syncytial Layer of zebrafish at Epiboly stage and thorough *in vitro* validation, would likely provide an answer on Nrz kinase identity.

Article 2 – Understanding the role of BCL2L10/Nrh in tumor progression

Introduction to Article 2

As reported in Article 1, we were able to highlight a novel mechanism of IP3R1-mediated calcium release regulation by a protein from the Bcl-2 family. Considering the involvement of BCL2L10/Nrh in human diseases, we thought that the validation that Nrh acts in the same way would bring a new understanding to Nrh contribution in pathologies. At first, we determined the expression of Nrh in a series of well-established cancer cell lines, to isolate a model cell line expressing Nrh at a sufficient level to better investigate its function. In a second time, we studied Nrh subcellular localization in our chosen model. This point seems critical, as Nrz has been shown to display a dual mitochondrial and ER localization in the zebrafish embryo (Popgeorgiev et al., 2011). In fact, Nrh localization has been investigated in recent studies using ectopically overexpressed Nrh, but the actual localization of the endogenous protein has yet to be determined (Van de Krooij et al., 2013).

To assess Nrh role in the different cellular compartments, we constructed several Nrh mutants with different subcellular localization, either replacing its native TM by cb5 ER-targeting domain, ActA mitochondrial-targeting domain, or removing the TM domain. We overexpressed these constructs into HeLa cells, which actually express some endogenous Nrh, but are much easier to manipulate for transient transfections than most breast cancer cell lines. We also thought to generate Nrh mutants having lost their ability to bind pro-apoptotic Bax/Bak, either by deletion of the hydrophobic groove or by single point mutation. These mutants are expected to demonstrate whether the observed effects are dependent or independent on BH3 binding.

The human IP3R1 receptor was subdivided into several functional fragments, according to the strategy used with zebrafish IP3R1 (See Article 1). It was expected that, similarly to Nrz, Nrh would interact with the IP3 Binding Domain of IP3R1 via its BH4 domain.

Finally, we envisioned a peptide-based approach to inhibit Nrh activity, as discussed above (See **Discussion relative to Article 1**). Actually, such an Nrh inhibitor may be used to screen a subset of cancer cell lines, and test their dependency on Nrh for survival. This may help to demonstrate that Nrh not only contributes to tumor progression or chemoresistance in those cell lines, but could also prove to be a worthy target to consider for cancer therapy.

Bcl-2 homolog BCL2L10/Nrh controls cell death and tumor progression at the Endoplasmic Reticulum

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Abstract

Apoptosis, also called "Programmed Cell Death", plays a pivotal role in many biological processes and pathologies. The B-cell lymphoma 2 (Bcl-2) proteins, whose expression is often altered in tumor cells, are the main regulators of apoptosis.

Among this family, the actual physiological function of the human apoptosis inhibitor Nrh, also referred to as BCL2L10 or Bcl-B, remains elusive.

Although in most healthy tissues the Nrh protein is nearly undetectable, clinical studies have shown that Nrh expression is correlated with poor prognosis in breast and prostate carcinomas. Recently, our group has uncovered that the Nrh ortholog in zebrafish, Nrz, was found to regulate calcium homeostasis through an interaction with the Ligand Binding Domain (LBD) of the Inositol-1,4,5-triphosphate receptor (IP3R) type-I Ca²⁺ channel (Bonneau et al., Science Signaling 2014).

Here we showed that Nrh interacts with the LBD of IP3Rs via its BH4 (Bcl-2 Homology 4) domain, which is critical to regulate intracellular Ca²⁺ trafficking and cell death. Actually, this interaction seems to be unique among the Bcl-2 family, and sets Nrh as the only Bcl-2 homolog to negatively regulate apoptosis by acting exclusively at the Endoplasmic Reticulum. Furthermore, we showed that disruption of the Nrh/IP3Rs complex primes Nrh-dependent cells to apoptotic cell death and enhances chemotherapeutic drug efficiency in breast cancer cell lines.

Together our results bring a new insight on the role of Nrh regarding its contribution to human diseases. Finally, our work support the idea that the Nrh BH4 domain is critical to understand its cytoprotective function. More generally speaking, anti-apoptotic Bcl-2 regulators BH4 domains might be valuable targets in order to devise new therapeutic strategies.

Introduction

Bcl-2 proteins have long been known as regulator of the mitochondrial pathway of Apoptosis (Chipuk et al., 2010). However, there is growing evidence supporting their involvement in various cellular processes, through a cellular localization outside the canonical mitochondrial residence (Schinzel et al., 2004). Indeed, the anti-apoptotic Bcl-2 proteins have been observed to regulate a variety of processes such as cell cycle, cell metabolism, cell motility as well as Ca2+ homeostasis (Bonneau et al., 2013). The latter process was linked to the direct interaction of several Bcl-2 proteins with IP3 receptors (IP3Rs), intracellular ion channels tightly regulating Ca2+ signaling at the Endoplasmic Reticulum (ER) membrane (Mikoshiba, 2007). Of note the N-terminal Bcl-2 Homology 4 (BH4) domain of Bcl-2 proteins was reported to be involved in such interactions (Monaco et al., 2012a; Rong et al., 2008).

In contrast, Bcl-2 proteins have distinct interaction sites on the IP3 receptors. Bcl-2 has been reported to interact mainly with the Modulatory and Transducing Domain II (MTD II) but weakly with the Coupling Domain (CD), whereas Bcl-xL and Mcl-1 have been reported to interact mainly with the CD and Bcl-xL interacting weakly with the MTD II domain in addition (Monaco et al., 2012b; Eckenrode et al., 2012).

Unlike these Bcl-2 homologs, the apoptosis regulator Nrz, was reported to interact instead with the IP3R1 Ligand Binding Domain (IP3R1-BD) in zebrafish.

This interaction uncovered a novel Nrz role as a regulator of Ca2+ homeostasis and actin cytoskeleton dynamics during early development (Arnaud et al., 2006; Popgeorgiev et al., 2011; Bonneau et al., 2014). Indeed, Nrz was shown to decrease the binding of IP3, the endogenous IP3R1 ligand, in a non-competitive manner.

These findings have shed a new light the human counterpart of Nrz, referred to as Nrh, BCL-B or BCL2L10.

Although described as a canonical anti-apoptotic Bcl-2 member (Inohara et al., 1998; Song et al., 1999; Aouacheria et al., 2001), Nrh *in vivo* relevance has been previously dismissed by the following observations: (1) absence of phenotype displayed from *nrh* knockout mice (Russel et al., 2002). (2) limited intrinsic capacity to inhibit Bax/Bak-dependent apoptosis (Rautureau et al., 2010). (3) restricted tissue expression pattern (Inohara et al., 1998; Song et al., 1999).

In fact, Nrh was only detected in B-cells (Luciano et al., 2007), and oocytes where it was shown to be a prosurvival factor (Guillemin et al., 2009).

More recently, *nrh* was reported to be overexpressed in neoplastic pathologies such as breast, prostate, lung cancer as well as myelodysplatic syndromes and acute myeloid leukemia (Krajewska et al., 2008; Cluzeau et al., 2012). In such diseases, *nrh* expression was correlated to poor prognosis, and in the case of myelomas, was also associated with resistance to azacytidine.

Given the input from both of these clinical studies and the previous results on Nrz, we sought to revisit Nrh contribution to pathology in the context of breast cancer.

Here, we report that Nrh is expressed in a subset of mammary cancer cell lines, and solely present at the ER, unlike other Bcl-2 proteins. We demonstrate that Nrh inhibits Ca2+ release and apoptosis through its interaction with the ligand binding domain IP3R1 and IP3R3 calcium channels. In addition, we used a peptide-based strategy to inhibit IP3Rs/Nrh interaction and reverse Nrh anti-apoptotic function. Actually, we show here that disruption of the IP3Rs/Nrh complex increases sensitivity to an array of chemotherapeutic agents. Together, our results show that Nrh may not only be a *bona fide* biomarker for chemoresistance, but could be also a potent target for anticancer therapy.

Results

Nrh localizes at the ER and contributes to apoptosis resistance

As an antiapoptotic factor, Nrh might represent a target of interest for anticancer therapy. However, to date Nrh was reported to be expressed in a limited number of cell types and tissues. In order to evaluate its possible implication in breast cancer, we analyzed Nrh expression levels in a series of breast cancer cell lines by western blotting. As shown in Fig. 1A, Nrh was found to be expressed in most of the analyzed cell lines, as well as in cervix cancer HeLa cells; however, the Nrh protein was not detected in the MCF10A cell line.

With respect to their subcellular localization, Bcl-2 homologs, including Nrz, the zebrafish ortholog of Nrh, are found both at the mitochondria and the ER. To determine the subcellular localization of Nrh we used the MDA-MB-231 and CAL51 cell lines, in which Nrh was found to be highly expressed. Interestingly, we observed that Nrh was localized at the ER but not at the mitochondria, suggesting that it could have a specific role in the ER compartment (Fig. 1B; supplementary Fig. 1A). To address this point, we analyzed the effect of Nrh silencing on the cell response to thapsigargin, an inhibitor of the SERCA Ca2+ pump that triggers massive Ca2+ release from the ER into the cytosol, leading to cell death.

In fact, in contrast to staurosporine, thapsigargin has a limited death-inducing effect in MDA-MB-231 cells. However, in Nrh-silenced cells (Fig. 1 C), thapsigargin triggered massive cell death as measured by caspase-3 and 7 activity (Fig. 1 D-E; supplementary Fig. 1B-E). Thus Nrh seems to be involved in a mechanism preventing cell death due to Ca2+ overload, following thapsigargin treatment.

ER-targeted Nrh prevent apoptosis independently of its BH3-binding pocket

To assess the importance of Nrh subcellular localization in this effect, we measured the capacity of Nrh chimeric proteins, addressed at either the ER or the mitochondria to sensitize the HeLa cells to thapsigargin, as compared to native Nrh (Fig. 2A, B & Supplementary Figure 2). As shown in Fig. 2C, Nrh-cb5 (ER-addressed), contrary to Nrh-Acta (mitochondria-addressed), prevented caspase activation with the same efficacy as native Nrh, following thapsigargin treatment. Thus the ability of Nrh to prevent thapsigargin-induced cell death seems to depend on its localisation at the ER. On the other hand, it should be noted that, mitochondria-addressed Nrh but not ER-addressed Nrh was found to prevent staurosporine-induced cell death (Fig. 2D). Interestingly, deletion of Nrh BH4 thwarted Nrh-mediated protection from both thapsigargin and staurosporine-induced apoptosis (Fig.2C-D).

Multidomain Bcl-2-like apoptosis inhibitors are known to trap the BH3 domains of apoptosis accelerators such as Bax and Bak, which prevent their insertion in the outer mitochondrial membrane and subsequent release of cytochrome c, leading to caspase activation and cell death.

To evaluate the extent to which the ability of Nrh to prevent cell death depended on such BH3-dependent interactions, we analyzed the effet of two Nrh mutants having lost their ability to bind to Bax or Bak. These mutants, namely Nrh G85A and Nrh 1-97, respectively bear a point mutation, hindering BH3 binding (Gurudutta et al., 2005; Robert et al., 2012), or a truncation at the C-terminus end, resulting in the same loss of function (Bonneau et al., 2014). In fact, both mutants were found to prevent caspase activation after thapsigargin treatment, like native Nrh (Fig. 2E-G). Thus at the level of the ER, the anti-death activity of Nrh does not seem to depend on complex formation with Bax-like death accelerators.

Nrh interacts with the IP3-binding domain of IP3R1 and IP3R3 to inhibit calcium signals

It was reported that the zebrafish ortholog of Nrh interacts with the IP3R1 calcium channel and controls the release of Ca2+ from the ER into the cytosol (Bonneau et al., 2014). We therefore investigated the ability of Nrh to interact with IP3R1. As shown in Fig. 3A&B, Nrh co-immunoprecipitated with the IP3 binding domain as well as the MTDII domain of IP3R1. Moreover, the BH4 Nrh deletion mutant lacking the N-terminus region was unable to bind to IP3R1, indicating that the N-terminus BH4 domain is critical for this interaction (Fig. 3C). It should be noted that Nrh interacts in fact with IP3R1 and IP3R3, but not IP3R2 IP3 binding domains (Fig. 3D).

Actually, full-length Nrh, as opposed to deltaBH4 Nrh, was found to prevent Ca2+ release from the ER following Histamine treatment in HeLa cells (Fig. 3E, F). Using the same experimental system, it was found that the two Nrh mutants that have lost the capacity to bind to Bax, prevented ER Ca2+ release with the same efficiency as native Nrh (Fig. 3 G, H). Therefore, the effect of Nrh on the relase of Ca2+ from the ER appears to depend on its interaction with IP3R1, but not with the Bax-like proteins.

A Nrh-derived 23 amino-acids motif is able to disrupt the IP3Rs/NRH interaction

Together our data suggest that Nrh may prevent cell death by lowering IP3R1 and IP3R3 channels permeability to Ca2+ ions. Thus in principle the disruption of the Nrh/IP3Rs complexes should sensitize the cells to death-inducing stimuli acting on Ca2+ homeostasis. Therefore, we analysed the ability of overlapping peptides corresponding to the BH4 domain of Nrh to disrupt the Nrh/IP3R1 complex. To this end we performed a series of co-IPs in cells transfected or not with plasmids expressing BH4-derived peptides. As shown in Fig. 4A&B, expression of the Nrh 1-23 peptide (corresponding to the first 23 residues of Nrh, encompassing the BH4 domain), and to a lesser extent the 5-23 peptide, were found to compromise complex formation; the other analyzed BH4 truncations seemed unable to do so (Fig. 4A, B). In a second step, we performed a series of point mutations scanning the BH4 sequence and showed that residues R6 and Y16 were critical for disrupting the Nrh/IP3R1 complex (Fig. 4C). In fact, as shown in Fig. 4D&E expression of the Nrh 1-23 peptide, as opposed to the Nrh 1-23 Y16F mutant, was found to enhance histamine-triggered Ca2+ release, and ultimately the cell response to

thapsigargin as measured *via* caspase activity (Fig. 4F). At the molecular level, we checked if the Nrh 1-23 peptide could compete with full length Nrh for IP3R1 binding. To this end we carried out a series of streptavidin pull down assays using a biotinylated Nrh 1-23 peptide. Amazingly, contrary to full-length Nrh (see Supplementary Figure 4), the Nrh 1-23 peptide alone was not found to interact either with the IP3R1 BD or with BECNI, a known interactor of Nrh (Robert et al., 2012) used here as a control. In contrast, Nrh 1-23 did interact with the Nrh protein itself, suggesting that disruption of the Nrh/IP3R1 complex might be due to association with Nrh but not IP3R1. Thus the Nrh 1-23 peptide does not seem to act as a decoy at the Nrh binding site on IP3R1, but instead binds directly Nrh to prevent Nrh/IP3R1 complex formation (Fig. 4G-H). Moreover, as expected, the mutation of R6 and Y16 residues of the Nrh 1-23 peptide abrogates its interaction with full-length Nrh. It should be noted that no interaction was detected with zebrafish Nrz, the closest functionally characterized Nrh ortholog (Fig. 4I-J).

Disruption of the endogenous IP3Rs/NRH interaction sensitizes cell to apoptosis

On the basis of these results, we investigated if a synthetic membrane permeable peptide (Nrh 1-23) could have similar effects, compared to transfected expression vectors. To this end, an FITC tag with a TAT sequence and a short spacer were added at the N-terminus end of the Nrh 1-23 peptide (TAT Nrh 1-23 peptide), the Y16F mutant being used as a control (TAT Nrh 1-23 Y16F peptide), see Fig. 5A. Of note, both peptides were found to adopt an alpha helical conformation in solution (Fig. 5B). Co-immunoprecipitation experiments performed on cell extracts incubated or not with the peptides showed that TAT Nrh 1-23, but not TAT Nrh 1-23 Y16F, was able to destabilize the Nrh/IP3R1 complex in a similar manner as previoulsy observed using expression vectors (Fig. 5C).

Next we investigated if the TAT Nrh 1-23 peptide could affect the formation of the complex *in situ*. To this end we used proximity ligation assays (PLA) to detect the endogenous Nrh/IP3R1 complex in non-transfected cells, expected to express physiologically relevant levels of Nrh.

To prevent trapping of the internalized peptide in the endosomes, we used a light source at 488 nm to excite the fluorophore FITC and induce endosomal leakage (see Muthukrishnan et al., 2012), increasing cytosolic availability and thus finely tuning TAT Nrh 1-23 peptide activity (Supplementary Fig. 5). In such conditions, as shown in Fig. 5D&E, whereas the PLA was positive in control cells (vehicle) as well as in cells incubated with the TAT Nrh 1-23 Y16F control peptide, no signal could be detected any longer in cells incubated with TAT Nrh 1-23. Together, these observations demonstrate that the TAT Nrh 1-23 peptide actually disrupts the Nrh/IP3R1 complex *in situ*.

Then, the effect of the peptide on the cell response to thapsigargin was investigated using the FLICA assay; as shown in Fig. 5F, thapsigargin triggered caspase activation in cells incubated with TAT Nrh 1-23 but not with the Y16F mutant. Thus sensitization to thapsigargin is closely related to the ability of the peptide to destabilize the Nrh/IP3R complex.

Taken together these data support the idea that the disruption of the Nrh/IP3R1 complex by the Nrh 1-23 peptide may enhance the cell response to death-inducing agents, in particular those agents acting on Ca2+ homeostasis.

Disruption of the IP3Rs/NRH interaction enhances response to chemotherapeutic agents

Then, we analyzed the cell response to a series of clinically approved molecules. In this study, we used a dimeric peptide. Indeed, it was recently reported that a dimeric form of the TAT peptide called dfTAT displayed enhanced endosomal escape capabilities (Erazo-Oliveras et al., 2014). Taking advantage of these findings, we sought to generate a more practical tool allowing to check sensitization to cell death on a broader range of apoptotic inducers and cell lines (Fig. 6A).

At first, we validated the activity of the newly formulated dfTAT Nrh 1-23 on the Nrh/IP3R1 BD interaction (Fig.6B). We used an Nrh high expressing cell line (MDA-MB-231), an Nrh low expressing cell line (MCF10A), as well as an Nrh Knock-out cell line (MDA-MB-231 Nrh KO) to assess the specificity of dfTAT Nrh 1-23 (Fig. 6C). Actually, MCF10A cells were found to be more resistant to thapsigargin, compared to MDA-MB-231. However, dfTAT Nrh 1-23 was only found to be able to circumvent thapsigargin resistance in MDA-MB-231 (high Nrh levels), but not in MCF10A (low Nrh levels) or MDA-MB-231 Nrh KO, see Fig. 6D-F, indicating that the observed sensitization was not an "off target" effect of the peptide.

As shown in Fig. 6G-H, the peptide, but not the mutant one, enhances apoptosis induced by high doses of etoposide (50 μ M) and doxorubicine (5 μ M). Moreover, at lower doses (4 μ M and 0.4 μ M respectively), which might be more clinically relevant, the peptide still acts as an efficient enhancer in the MDA-MB-231 cell line (Fig. 6I-J). It should be noted that in these latter conditions, cell density was used as a read-out instead of apoptosis, since caspase-3 and 7 were not found to be activated, at least in the time frame used. Thus even in « mild conditions » the peptide appears to be able to compromise the growth of cancer cells in culture. We further validated our results in the CAL-51 cell line, although its response to chemotherapeutic agents seemed to differ from the MDA-MB-231 cell line. Indeed, we showed that in these cells, the Nrh 1-23 peptide is a strong enhancer of azacytidine (25 μ M) and doxorucibine (0.1 μ M) activity (Fig. 6K-L), but not etoposide (data not shown).

Disruption of the IP3Rs/NRH interaction compromises tumor progression

Together, the previous data show that the Nrh 1-23 peptide enhances the cell response to several agents currently used in the clinic for anticancer chemotherapy. The next step was thus to check if this peptide could actually affect tumor progression.

To address this point we analyzed the effect of TAT Nrh 1-23 on colony formation in soft agar. In this assay, MDA-MB-231 cells were incubated in presence or not of the peptide (1-23 or Y16F mutant), with or without etoposide (1 μ M), and grown in soft agar. Fig. 7A and 7B show that the TAT Nrh 1-23 peptide efficiently prevents the development of the colonies in conditions where etoposide alone has a limited effect. It should also be noted that the peptide alone appeared to have no effect. Taken together, these data demonstrate the existing synergy between etoposide and the Nrh 1-23 peptide with respect to colony formation in agar by breast cancer cells.

Discussion

Although the *nrh* gene was characterised more than 15 years ago, its roles remain largely enigmatic. One of the reasons for this may possibly be that the KO of *diva/boo*, the mouse ortholog of *nrh*, gives no apparent phenotype (Russel et al., 2002). In human, *nrh* pattern of expression is somewhat limited, although there is evidence that *nrh* could play a specific role in the maturation of the oocytes (Guillemin et al., 2009). In the context of cancer, *nrh* expression have been correlated to chemoresistance and poor prognosis, suggesting that Nrh might be a promising therapeutic target.

The Nrh protein was reported to be an inhibitor of apoptosis and autophagy. We have recently showed that the zebrafish ortholog of Nrh was able to control cytoskeleton dynamics by interacting with the caclcium channel IP3R1. This prompted us to evaluate the extent to which Nrh could also play a role at the level of the ER. Interestingly, the observed ER localisation of Nrh supports such a possibility. This hypothesis was reinforced by the fact that Nrh was found to interact with IP3R1 via its BH4 domain and decrease the release of Ca2+ from the ER.

Unlike zebrafish Nrz, the silencing of human Nrh does not appear to affect cell migration. In normal culture conditions, Nrh silencing does not compromise cell viability either. In contrast, in stress conditions, Nrh silencing dramatically increases the cell response to death-inducing agents by enhancing the release of Ca2+ from the ER. Thus Nrh is the only Bcl-2 homolog that acts at the ER, but not at the mitochondria, to prevent cell death in stress conditions.

The fact that Nrh silencing does not affect apoptosis on normal conditions may explain in part the absence of detectable phenotype in *diva/boo* KO mice. Thus the exact function of Nrh in physiological conditions remains to be determined. In the present work, we evaluated if Nrh could represent a valuable target for anticancer therapy.

The fact that Nrh was exclusively found at the ER prompted us to check if the disruption of the Nrh/IP3R1 complex could be an interesting approach. Actually, the observation that disrupting this complex using the BH4 derived peptide sensitizes the cells to apoptosis confirmed this assertion. Of note, the Bcl-2/IP3R2 interaction was reported to be another target of interest in the context of leukaemia.

Actually, a short IP3R1-derived peptide was showed to be able to trigger apoptosis by disrupting Bcl-2/IP3Rs complexes (Akl et al., 2013). Here we show that a peptide derived from the BH4 domain of Nrh has similar properties. Moreover, the use of a peptide derived from a single Bcl-2 homolog may enhance its specificity and prevent unwanted secondary effects.

Surprisingly, the BH4 peptide appears to disrupt the Nrh/IP3R1 complex through binding Nrh protein but not the IP3R1 BD as could be expected at first. This result illustrates again the advantages of isolating novel agents targeting the Bcl-2 family of proteins for anticancer therapy. However, the exact region of Nrh that is recognized by the BH4 peptide remains to be characterized. The possibility exists that the binding of the peptide induces an Nrh conformational change, leading to the disruption of the Nrh/IP3R1 complex. It would be of interest to evaluate if this peptide affects the subcellular localization of Nrh and its interaction with other protein partners. Moreover, our data show that the BH4 peptide decreases the tumor potential of breast cancer cells. It would be worth investigating if it has similar effects in other types of cancer overexpressing Nrh, including leukaemia.

Finally, most therapeutic strategies devised to inhibit pro-survival Bcl-2 proteins have been focused on the BH3 domain. Recently, the newly found cytoprotective function of Bcl-2 BH4 have been exploited to successfully develop new drug candidates (Lavik et al., 2015; Han et al., 2015). This approach offers the opportunity of better specificity, but also lower residual toxicity. For instance, Bcl-xL inhibitors are known to interfere with platelets survival, causing life threatening side effects in mice, such as thrombocytopenia (Mason et al., 2007).

Our present results tend to support the concept that pro-survival Bcl-2 BH4 domains should be investigated as prime targets for drug development in the future.

Material and Methods

siRNA, peptides, reagents and antibodies

Interfering siRNA against Nrh were purchased from IDT DNA (HSC.RNAI.N020396.12.3, HSC.RNAI.N020396.12.4, HSC.RNAI.N020396.12.5, and HSC.RNAI.N020396.12.6).

The following drugs were used to treat cells: Thapsigargin (BML-PE180, Enzo), Staurosporin (S4400, Sigma), Histamine (H7125, Sigma), Doxorubicin (44583, Sigma), Etoposide (E1383, Sigma), Azacytidine (A2385, Sigma).

The following antibodies were used: rabbit IP3R1 (PA1-901, Thermo Scientific) mouse IP3R1 (SC-271197, Santa Cruz Biotechnology), rabbit FLAG (F-7425, Sigma-Aldrich), mouse FLAG (F-3165, Sigma-Aldrich), rabbit HA (Ab75640, Abcam), mouse HA (MMS-101P, Covance), mouse Vinculin (SC-55465, Santa Cruz Biotechnology), mouse Actin (MAB1501, Millipore), mouse F0F1 ATPase (612518, BD Bioscience), rabbit Nrh (#3869, Cell Signaling Technology), rabbit Nrh (#3695,

Biovision), rabbit Calnexin (#2679, Cell Signaling Technology), mouse Tubulin (SC-23950, Santa Cruz Biotechnology).

Peptides with the following sequence: FITC TAT-Nrh 1-23 FITC (RKKRRQRRRGGSGGADPLRERTELLLADYLGYCARE), TAT-Nrh 1-23 Y16F (RKKRRQRRRGGSGGADPLRERTELLLADFLGYCARE), TMR dfTAT-Nrh 1-23 (CKRKKRRQRRRGGSGGADPLRERTELLLADYLGYAARE), TMR dfTAT-Nrh 1-23 Y16F (CKRKKRRQRRRGGSGGADPLRERTELLLADFLGYAARE) were chemically synthetized by Covalab (Lyon, France). Biotin Nrh 1-23 (MADPLRERTELLLADYLGYCARE) and Biotin Nrh 1-23 R6A Y16F (MADPLAERTELLLADFLGYCARE) were obtained from the C3D plateform (CRCL, Lyon, France).

Vectors and constructions

Nrh constructions and IP3Rs fragments were cloned into pCS2+ vector with ClaI and XhoI restriction sites. When indicated, the constructions were cloned into pCS2+ cb5 or pCS2+ ActA, containing downstream of the MCS either human cytochrome cb5 ER-targeting domain or ActA (from *L. monocytogene*) mitochondria-targeting domain nucleic sequences between XhoI and XbaI restriction sites. HA-Beclin1 plasmid was obtained from addgene (#24399).

Immunoprecipitation & Pull-Down Assays

For endogenous immunoprecipitation experiments $6x10^6$ HeLa cells were transfected with the indicated vectors and grown for another 24 h. Cells were lysed in TNE buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA (pH 7.4), supplemented with 1 mM β -glycerophosphate, 1 mM sodium orthovanadate, 0.1 mM sodium pyrophosphate, 0.2 % NP-40 and a protease inhibitor cocktail (Roche)). Extracts were precleared with protein G-Sepharose beads (Sigma) for 2 h at 4°C, then incubated overnight with 5 μ g IP3R1 primary antibody. Afterwards, extracts were incubated with protein G-Sepharose beads for 3 h. Immunoprecipitated fractions were washed three times with TNE buffer, then analysed by immunoblotting. For HA and Flag immunoprecipitation, 1x10⁶ HeLa cells were seeded 24 h before, then transfected with the indicated vectors and grown for another 24 h. 2 μ g of primary antibody HA rabbit or FLAG mouse was used.

For pull-down experiments, 6x10⁶ HeLa cells were transfected with the indicated vectors and grown for another 24 h. Lysates were pre-cleared with protein Streptavidin beads (Dynabeads MyOne Streptavidin T1, Thermo) for 2 h at 4°C split in two and 5µg of purified biotin-coupled Nrh 1-23 WT or Nrh 1-23 R6A/Y16F peptides were added when used.

Subcellular Fractionation

All steps were carried out at 4°C if not otherwise stated. $20x10^{6}$ MDA-MB-231 cells were resuspended in 1mL cold MB buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES (pH 7.5)) and disrupted using a dounce tissue grinder. Extracts were centrifugated at least five time at 1500 x g for 5 min to eliminate nuclei, then centrifugated at 10600 x g for 10 min. The pellet containing mitochondria was washed twice in MB buffer then resuspended in TNE buffer. The supernatant was then centrifuged at 100000 x g for 1 h. The supernatant containing the cytosolic fraction was conserved and the pellet containing ER membranes resuspended in TNE buffer for immunoblotting.

Proximity ligation assay

Cells grown on coverslips into 12-well plates were fixed in methanol for 2 min, and washed twice in PBS. Samples were saturated using the blocking solution, then incubated for 1 h at 37°C with a couple of primary antibodies (rabbit Nrh Biovision and mouse IP3R1 Santa Cruz). After washes, the PLA minus and plus probes (containing the secondary antibodies conjugated with complementary oligonucleotides) were added and incubated 1 h at 37°C. After the ligation of oligonucleotides, the addition of nucleotides and polymerase allowed amplification by rolling-circle amplification (RCA) reaction using the ligated circle as a template, during an incubation of 100 min at 37°C. The amplification solution contained fluorescently labeled oligonucleotides that hybridized to the RCA product. Afterwards, the samples were mounted with Duolink II Mounting Medium containing Dapi, and then analyzed on fluorescence microscope.

Intracellular Ca²⁺ measurements

HeLa cells cultured in NuncTM Labtek® chambered coverglass were incubated with 5 μ M of FluoForteTM Ca²⁺ probe in a Ca²⁺-free Balanced Salt Solution (BSS) (121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 6 mM NaHCO₃, 5.5 mM D-glucose, 25 mM HEPES, pH 7.3) for 45 min at 37°C. Time-lapse fluorescence values were collected using a Zeiss LSM 780 confocal microscope. After 10 seconds of measurement, 100 μ M histamine in Ca²⁺-free was added.

Apoptosis induction and assay

Cells were treated with either staurosporine (1 μ M for 8 h), thapsigargin (ranging from 0.1 to 10 μ M, for 24 to 36 h), etoposide (50 μ M for 36h), or doxorubicin (5 μ M for 24h).

For kinetic experiments, 1x10⁴ cells were seeded in a 96-well plate 24 h before transfection. Cells were transfected with 50 nM of different siRNAs using Lipofectamine ® RNAiMAX reagent (Invitrogen), then incubated for another 48 h. Apoptosis was assessed through observed caspase activity using CellPlayerTM Kinetic Caspase-3/7 Apoptosis reagent (Essen Bioscience) diluted 1:2000. Images were acquired using an IncuCyte ZOOM TM. Caspase activity was corrected to cell density to obtain % of apoptotic cells.

For endpoint experiments, 1×10^5 cells were seeded in a BD Falcon chambered culture slide 24 h before transfection. Cells were transfected with 0.5 µg of pCS2+-EGFP vector mixed with 1 µg of the indicated vectors using XtremeGene HP transfection reagent (Roche), then grown for another 24 h. Apoptosis was assessed at the end of the corresponding drug treatment using SR-FLICA® Poly Caspase Assay staining (ImmunoChemistry Technologies). Images were acquired using a Nikon NiE microscope and green transfected cells positive for FLICA staining were counted with a custom-made macro on the ImageJ software to calculate % green cells displaying caspase activity.

Circular Dichroïsm

Briefly, FITC TAT-Nrh 1-23 and FITC TAT-Nrh 1-23 Y16F were diluted in peptide solvant [H2O-TFA 0.1%] at 80µM final concentration, with 20% 2,2,2-Trifluoroethanol (05841, Sigma). Circular dichroïsm spectra were recorded using a Chirascan CD Spectrometer (Applied Photophysics).

Proliferation assay

For MDA-MB-231 cell line, $4.5x10^3$ cells were seeded in a 96-well plate 24h before incubation with dfTAT peptides. MDA-MB-231 cells were treated with 4 μ M etoposide or 0.04 μ M doxorubicin. For CAL51 cell line, $8x10^3$ cells were seeded in a 96-well plate 24h before incubation with dfTAT peptides. CAL51 cells were treated with 25 μ M azacytidine or 0.1 μ M doxorubicin. Cell density was assessed and images were acquired using an IncuCyte ZOOM TM over a 96h time frame.

Agar colony formation assay

Briefly, 1.5×10^4 MDA-MB-231 cells were seeded in a 24-well plate, then incubated for 24h at 37°C. Cells were incubated 1 h at 37°C with 10 µM dfTAT peptides in Ca²⁺-free BSS, then washed and incubated another 24 h with or without 1 µM etoposide. Cells were then trypsinated, counted, and 2.5×10^3 cells per condition grown for 14 days into DMEM 10% FBS 0.45% agar top layer (1 mL final volume), with DMEM 10% FBS 0.75% agar bottom layer (1 mL final volume) in a 12-well plate. Every 3 days 200 μ L fresh media (DMEM 20% FBS) was added, and colonies were manually counted at the end of the 14 days growth.

Statistical Analysis

Statistical significance was analyzed using the Student t-test; p<0.05 was considered significant.

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Fig. 1. Nrh localizes at the ER and contributes to apoptosis resistance

(A) Immunoblotting of endogenous Nrh level in a subset of human mammary cell lines. (B) Immunoblotting of endogenous Nrh from total fraction (Total), mitochondrial fraction (Mito), reticular fraction (ER), or cytosolic fraction after 100000G centrifugation (S100), prepared from MDA-MB-231 breast carcinoma cell line. (C) Immunoblotting of endogenous Nrh protein (100 μ g lysate) transfected by several siRNA against Nrh at 50 nM for 48 h. (D) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 cells transfected with siRNAs and treated with 10 μ M thapsigargin displayed at 36h (N=3). (E) Kinetic apoptosis assay (Caspases 3 and 7 activity) in MDA-MB-231 cells transfected with siRNAs and treated with siRNAs and treated with 1 μ M staurosporine displayed at 8h (N=3). ***, p<0.001 ; **, p<0.01 ; *, p<0.05 ; n.s. p>0.1 (Student t-test)









D













Fig. 2. ER-targeted Nrh prevent apoptosis independently of its BH3-binding pocket

(A) BCL2L10/Bcl-B/Nrh (Nrh-WT) structure schematic: BH domains (Bcl-2 Homology domain) and TM (Transmembrane domain), along Nrh constructions targeted to the mitochondria using ActA domain (Nrh-ActA), targeted to the endoplasmic reticulum (ER) using cb5 ER-anchoring domain (Nrh-cb5), or truncated of its BH4 domain (Nrh-dBH4). (B) Immunoblotting showing expression of the different Nrh constructs from (A) compared to empty vector (pCS2+). (C) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected by the Nrh constructs showed in (A) and treated with 10 μ M thapsigargin for 36h (N=3). (D) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected by the Nrh constructs showed in (A) and treated with 10 μ M thapsigargin for 36h (N=3). (D) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected by the Nrh constructs showed in (A) and treated with 10 μ M thapsigargin for 36h (N=3). (D) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected by the Nrh constructs showed in (A) and treated with 10 μ M thapsigargin for 36h (N=3). (D) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected by the Nrh constructs showed in (A) and treated with 10 μ M thapsigargin for 36h (N=3). (F) Immunoblotting showing expression of the BH1 domain (Nrh G85A) or deletion of half of the BH1 domain and the BH2 domain (Nrh 1-97). (F) Immunoblotting showing expression of the different Nrh constructs from (E). (G) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected by the Nrh constructs showed in (E) and treated with 10 μ M thapsigargin for 36h (N=3). ***, p<0.001 ; **, p<0.01 ; *, p<0.05 ; n.s. p>0.1 (Student t-test)
hIP3R1



Fig. 3. Nrh interacts with the IP3-binding domain of IP3R1 and IP3R3 to inhibit calcium signals

(A) Human Inositol 1,4,5-triphosphate receptor type 1 (hIP3R1) structure schematic, split into seven functional domain: SD (Suppressor Domain), IP3BD (IP3 ligand Binding Domain), MTD (Modulatory and Transducing Domain), CFD (Channel Forming Domain) and CD (Coupling Domain). (B) Immunoprecipitation experiment (IP) using HA antibody in HeLa cells transfected with a plasmid coding for HA-tagged hIP3R1 domains and Flag-Nrh. (C) Immunoprecipitation experiment using IP3R1 antibody in HeLa cells transfected with a plasmid coding for either Flag-Nrh or Flag-Nrh-dBH4. (D) Immunoprecipitation experiment using HA antibody in HeLa cells transfected with a plasmid coding for either Flag-Nrh or Flag-Nrh-dBH4. (D) Immunoprecipitation experiment using HA antibody in HeLa cells transfected with a plasmid coding for HA-tagged hIP3R1, hIP3R2 or hIP3R3 IP3 binding domains and Flag-Nrh. (E) Representative curves of calcium transients detected with the FluoForte cytosolic calcium dye after 100µM histamine stimulation in HeLa cells transfected by either empty vector (pCS2+), Nrh or Nrh dBH4 expressing vector. (F) Max peaks as recorded in (E) (N≥10). (G) Representative curves of calcium transients detected with the FluoForte cytosolic calcium dye after 100µM histamine stimulation in HeLa cells transfected by either empty vector (pCS2+), Nrh, Nrh G85A or Nrh 1-97 expressing vector. (H) Max peaks as recorded in (G) (N≥10). ***, p<0.001 ; **, p<0.01 ; **, p<0.05 ; n.s. p>0.1 (Student t-test)

А















Fig. 4. A Nrh-derived 23 amino-acids motif is able to disrupt the IP3Rs/NRH interaction

(A) Nrh BH4 overlapping constructions schematic. (B) Immunoprecipitation experiment using HA antibody in HeLa cells transfected with a plasmid coding for Flag-Nrh, HA-hBD and Flag-Nrh BH4 constructs with a 1:2 ratio. (C) Immunoprecipitation experiment using HA antibody in HeLa cells transfected with plasmids coding for Flag-Nrh, HA-hBD and Flag-Nrh BH4 point mutant constructs with a 1:2 ratio. (D) Endpoint apoptosis assay (SR-FLICA) in HeLa cells transfected with empty vector, or plasmids coding for Nrh 1-23, Nrh 1-23 Y16F or Nrh 1-23 C20A and treated with 10 μ M de thapsigargin for 24h (N=3). (E) Representative curves of calcium transients detected with the FluoForte cytosolic calcium dye after 100 μ M histamine stimulation in HeLa cells transfected by either empty vector (pCS2+), Nrh 1-23 WT, Nrh 1-23 Y16F or Nrh 1-23 C20A expressing vector. (F) Max peaks as recorded in (E) (N≥9). (G) Streptavidin Pull-down experiment with or without Nrh 1-23 chemically synthetized peptide with biotin coupling in HeLa cells transfected with a plasmid coding for Flag-Nrh, HA-tagged hBD-IP3R1 or HA-tagged BECN1. (H) Input protein lysates (30 μ g) from (G) Pull-down experiment. (I) Streptavidin Pull-down experiment with a plasmid coding for Flag-Nrh, HA-tagged hBD-IP3R1 or HA-tagged Nrh 1-23 R6A/Y16F chemically synthetized peptides with biotin coupling in HeLa cells transfected with a plasmid coding for Flag-Nrh, HA-tagged Nrh 1-23 or Nrh 1-23 R6A/Y16F chemically synthetized peptides with biotin coupling in HeLa cells transfected with a plasmid coding for Flag-Nrz. (J) Input protein lysates (20 μ g) from (I) Pull-down experiment. ***, p<0.01; **, p<0.01; **, p<0.05; n.s. p>0.1

A



 λ_{488nm}

Vehicle

Circular Dichroïsm (mDeg)



TAT-Nrh 1-23

Е

DAPI/PLA

_

DAPI/PLA/FITC





+ HA-hBD

+ Flag-Nrh

TAT-1-23 V10F TAT-1-23

110 kDa

40 kDa

25 kDa

40 kDa

25 kDa

F



TAT-Nrh 1-23

DAPI/PLA/FITC



Fig. 5. Disruption of the endogenous IP3Rs/NRH interaction sensitizes cell to apoptosis

(A) TAT-Nrh 1-23 synthetic peptide sequences, including TAT cell-penetrating domain, linker and Nrh 1-23 sequence without methionine. (B) Circular dichroïsm spectra of TAT-Nrh 1-23 and TAT-Nrh 1-23 Y16F (at 80 μ M in [H2O-TFA 0.1%-20% TFE]) showing alpha-helical structure signature. (C) Immunoprecipitation experiment using HeLa pooled lysates, either transfected with a plasmid coding for Flag-Nrh or HA-hBD and incubated along the Flag antibody with VEHICLE (peptide solvant [H2O-TFA 0.1%]), control peptide (TAT-Nrh 1-23 Y16F) or wild-type peptide (TAT-Nrh 1-23). (D) Proximity Ligation Assay (PLA) using FITC-labeled TAT-Nrh 1-23 peptides either photoactivated (λ 488nm [+]) or not (λ 488nm [-]), detecting the endogenous interaction between IP3R1 and Nrh in MDA-MB-231 cells. See supplementary Fig. 5. (E) Quantification of PLA experiments (N=3). (F) Endpoint apoptosis assay (SR-FLICA) in MDA-MB-231 cells using FITC-labeled TAT-Nrh 1-23 peptides either photoactivated (λ 488nm [+]) or not (λ 488nm [-]) and treated with 10 μ M de thapsigargin for 36h (N=3). ***, p<0.001 ; **, p<0.01 ; *, p<0.05 ; n.s. p>0.1 (Student t-test)



Fig. 6. Disruption of the IP3Rs/NRH interaction enhances response to chemotherapeutic agents

(A) dfTAT-Nrh 1-23 synthetic peptide sequences. (B) Immunoprecipitation experiment using HA antibody in HeLa cells transfected with a plasmid coding for Flag-Nrh or HA-hBD, incubated for 1h at 37°C in Ca²⁺-free BSS with Vehicle (peptide solvant [H2O-TFA 0.1%]), 10 μ M control peptide (dfTAT-Nrh 1-23 Y16F) or 10 μ M wild-type peptide (dfTAT-Nrh 1-23), washed twice and incubated for another 1 hour at 37°C. (C) Immunoblotting showing relative expression of endogenous Nrh in MDA-MB-231, MCF10A, CAL-51 and MDA-MB-231 Nrh KO cell lines. (D) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MCF10A cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 1 μ M thapsigargin, displayed at 24h (N=3). (E) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 Nrh KO cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 1 μ M thapsigargin, displayed at 24h (N=3). (E) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 Nrh KO cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 1 μ M thapsigargin, displayed at 24h (N=3).

(F) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 1 μ M thapsigargin, displayed at 24h (N=3). (G) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 50 μ M etoposide, displayed at 36h (N=3). (H) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 50 μ M etoposide, displayed at 36h (N=3). (H) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 5 μ M doxorubicin, displayed at 24h (N=3). (I) Kinetic cell proliferation assay of MDA-MB-231 cells incubated for 1h at 37°C with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 4 μ M etoposide, displayed at 96h (N=3). (J) Kinetic cell proliferation assay of MDA-MB-231 cells incubated for 1h with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 4 μ M etoposide, displayed at 96h (N=3). (J) Kinetic cell proliferation assay of MDA-MB-231 cells incubated for 1h with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 0.04 μ M doxorubicin, displayed at 96h (N=3). (K) Kinetic cell proliferation assay of CAL51 cells incubated for 1h with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 25 μ M azacytidine, , displayed at 96h (N=3). (L) Kinetic cell proliferation assay of CAL51 cells incubated for 1h with 10 μ M dfTAT peptides in Ca²⁺-free BSS and treated with 0.1 μ M doxorubicin, displayed at 96h (N=3). ***, p<0.001; **, p<0.01; **, p<0.05; n.s. p>0.1 (Student t-test)





Fig. 7. Disruption of the IP3Rs/NRH interaction compromise tumor progression

(A) Images of agar colony formation assay of MDA-MB-231 incubated for 1h with 10 μ M dfTAT peptides in Ca²⁺-free BSS, treated with 1 μ M etoposide for 24h, then grown on soft agar for another 14 days (B) Quantification of colonies found in agar at 14 days. ***, p<0.001 ; **, p<0.01 ; *, p<0.05 ; n.s. p>0.1 (Student t-test)



Fig. 8. Nrh regulates apoptosis at the ER

Nrh BH4 interacts with IP3R1 receptor in steady-state cells to negatively regulate calcium signaling. Upon Nrh downregulation or complex disruption by Nrh 1-23 peptide, a rise in cytosolic calcium might lead to calcium import from ER to mitochondria through VDAC (Voltage Dependant Anion Channel) and MCU (Mitochondrial Calcium Uniporter) channels. Excessive calcium entry into mitochondria may trigger mitochondrial outer membrane permeabilisation (MOMP), cytochrome c release and cell death.

Supplementary Figures



Supplementary Fig. 1. Nrh expression is restricted at the ER in CAL51 cell line and potentiates thapsigargin dosedependent effect in MDA-MB-231 cell line

(A) Immunoblotting of endogenous Nrh from total fraction (Total), mitochondrial fraction (Mito), reticular fraction (ER), or cytosolic fraction after 100000G centrifugation (S100), prepared from CAL51 breast carcinoma cell line. (B) Kinetic apoptosis assay (% of cells displaying Caspases 3/7 activity) in MDA-MB-231 cells transfected with siRNAs and treated with 10 μ M thapsigargin for 36h, (C) 1 μ M thapsigargin or (D) 0.1 μ M thapsigargin (N=3). (E) Representation % of cell percentage displaying Caspases 3/7 activity in function of LOG[thapsigargin] concentration using two siRNA against Nrh at 36h time point.







Supplementary Fig. 2. Subcellular localization of Nrh overexpressed constructions

(A) Immunoblotting of Flag-Nrh from total fraction (Total), mitochondrial fraction (Mito), reticular fraction (ER), prepared from of HeLa cells transfected Nrh, Nrh-cb5 or Nrh-ActA. (B) Immunofluorescence staining of Flag-Nrh with Alexa Fluor 561 nm in HeLa cells co-transfected Nrh, Nrh-cb5 or Nrh-ActA and ER-GFP reporter plasmid to show Nrh and Nrh-cb5 colocalization at the ER. Immunofluorescence staining of Flag-Nrh with Alexa Flour 488 nm in HeLa cells co-transfected Nrh, Nrh-cb5 or Nrh-ActA together with MitotrackerTM Red staining was performed to ensure Nrh-ActA localization at the mitochondria.



Supplementary Fig. 3. Nrh is the main Bcl-2 protein interacting with IP3R1 Binding Domain

Immunoprecipitation experiment using anti-HA antibody in HeLa cells transfected with a plasmid coding for either HA-tagged hBD-IP3R1 and Flag-Nrh, Flag-Bcl-2, Flag-Bcl-xL, Flag-Bfl-1, or Flag-Bcl-W.



Supplementary Fig. 4. Full-length Flag-Nrh interacts with GST-hBD IP3R1

GST pulldown experiment of 200 μ g cell lysate transfected with either pCS2+ or Nrh plasmid, co-incubated with 5 μ g of recombinant GST-hBD IP3R1 protein.



Supplementary Fig. 5. Full-length Flag-Nrh interacts with GST-hBD IP3R1

Schematic showing the protocol used to facilitate FITC-coupled TAT-Nrh-1-23 diffusion into the cytosol and escape through the endosomal barrier, with the help of a light irradiation at 488 nm.



Complementary Figure 1

Complementary Fig. 1. Nrh expression in healthy tissues

Immunohistochemistry staining of Nrh in healthy tissues micro-array, with Sigma BCL2L10 antibody diluted to 1/400. Strong expression can be observed in Kidney, moderate expression in Fallopian tube, Esophagus, Thyroid, Cerebral cortex, Cerebellum, and weak expression in Liver. No other tissue was shown positive to Nrh staining. Breast, Colon, Prostate Thymus and Lung are shown as negative control.

Discussion relative to Article 2

Nrh TM domain does not account for its exclusive ER localization

Until recently, BCL2L10/Nrh contribution to tumor progression was still misunderstood. Prior to our study, no investigation was made on the possible involvement of Nrh in breast cancer progression. We successfully identified three model cell lines, two high-expressing cell lines MDA-MB-231 and CAL51, and an Nrh low-expressing cell line MCF10A.

Thanks to subcellular fractionation, we have demonstrated that, at least at endogenous levels in breast cancer cell lines, Nrh is exclusively localized at the ER. We have also shown in supplementary Fig.2 that Nrh can be found, when overexpressed, at the mitochondrial membrane, even though it has an ER-targeting sequence (cb5). In addition, we observed that Nrh could localize to the ER and exert its protective function even deleted of its transmembrane C-terminal domain (data not shown). We have shown that in HeLa cells, enforced mitochondrial localization of Nrh hindered its protective effect against a thapsigargin challenge, a drug known to induce calcium-dependent cell death, by increasing cytosolic calcium level through inhibition of SERCA pump at the ER.

On this basis, it can be speculated that the TM domain of Bcl-2 proteins such as Nrh, has the ability to anchor the protein both at the mitochondria and the endoplasmic reticulum. However protein-protein interactions may be required to specifically address the protein to one compartment or the other.

Therefore, upon reaching the threshold of Nrh protein bound to a putative partner that directs its localization, the unbound fraction in excess may be redistributed to other membranes. This phenomenom might actually happen in conditions where the Nrh protein is ectopically overexpressed.

Nrh may prevent apoptosis indirectly by preventing ER-stress

Interestingly, the strongest thapsigargin effect on apoptosis was observed between 24h and 36h of treatment. This delayed response indicates that calcium may not directly trigger apoptosis in this case, but that alternatively massive calcium efflux might trigger an ER stress response, as many ER-located chaperones are dependent of calcium for optimal activity (Coe et Michalak, 2009).

The accumulation of unfolded proteins at the ER is known to cause a cell response referred to as the UPR (Unfolded Protein Response). UPR can activate safeguard mecanisms that promote cell survival by eliminating unfolded proteins and promoting the correct folding of proteins, or whenever necessary, trigger an apoptotic response through the expression of transcription factor CHOP (Hetz, 2012). CHOP is actually responsible for activating the transcription of pro-apoptotic BH3-only protein Bim and repressing Bcl-2 transcription (Hetz, 2012). A response time frame ranging from 24 to 36h for maximum effect depending on the cell line may in fact indicate a transcriptional response tied to thapsigargin effect.

Nonetheless in that case, Nrh would most likely acts upstream the UPR by suppressing IP3Rs calcium efflux, as Nrh 1-97 and G85A mutants having lost their activity to interact with Bax/Bak and BH3-only proteins can still protect from thapsigargin-mediated apoptosis.

Nrh might act as specific inhibitor of IP3R1 and IP3R3-mediated calcium signals

We have shown that Nrh interacts, via its BH4 domain, with IP3R1 IP3 BD and MTDII. We mainly considered the interaction between Nrh and IP3 BD as the primary mechanism regulating calcium efflux, based on IP3 BD importance in IP3R1-mediated calcium release and previous results on Nrh. Nevertheless, interaction with the MTDII domain, the same interaction domain as Bcl-2, should also be investigated although our preliminary results showed that such an interaction may not involve the Nrh BH4 domain (data not shown).

Amazingly, Nrh interaction with IP3 BD was observed with IP3R1 and IP3R3, but not IP3R2. Moreover, IP3R3 was the IP3R isoform mostly detected in the screened breast cancer cell lines, especially in MDA-MB-231 mainly expressing IP3R2 and IP3R3 (but also slight amount of IP3R1), and CAL51 expressing exclusively IP3R3 (data not shown). A recent study has in fact found a role for IP3R3 in breast tumor progression, to sustain cell growth in response to oestrogen stimulation (Szatkowski et al., 2010). Thereby, even if we studied Nrh interaction with IP3R1 in our work, from clues coming from Nrz study in zebrafish, interaction between Nrh and IP3R3 might be in fact more relevant in the context of breast cancer. Furthermore, IP3R2 seems to be the main IP3R isoform regulated by Bcl-2 in the context of diffuse large B-cell lymphoma (Akl et al., 2013; Ivanova et al., 2014; Vervloessem et al., 2015). Thus one can formulate the hypothesis from these publications that BCL2L10/Nrh might be specialized in the negative regulation of IP3R1/IP3R3-mediated calcium fluxes, whereas Bcl-2 would be specialized mainly in IP3R2 regulation.

Nrh BH4 domain is the first known isolated BH4 to bind an anti-apoptotic Bcl-2 protein

As we confirmed the involvement of the Nrh BH4 domain in the complex formation with IP3Rs in Article 2, we took the time to dissect the molecular basis of Nrh BH4 interface with IP3 BD. Interestingly, only the full 1-23 BH4 domain of Nrh, and to some extent Nrh 5-23, were found to inhibit Nrh binding to IP3 BD in competition experiments. In addition, Nrh 1-23 point mutation R6A and Y16F were shown to reduce the ability of the peptide to disrupt Nrh/IP3 BD interaction. However, much to our surprise, no direct interaction was detected between the Nrh 1-23 peptide and the IP3 BD. Using pull-down experiments, we further characterized that the Nrh 1-23 peptide was able to bind Nrh itself, but not IP3R1 BD or Beclin-1, a known partner of Nrh. This point seems highly interesting and raises many questions still to be answered, such as the interaction site of Nrh BH4 within full-length Nrh, or ability of full-length Nrh to dimerize through its N-terminus. Nevertheless, when overexpressed in HeLa cells, the Nrh 1-23 peptide was able to inhibit Nrh action on calcium fluxes and circumvent Nrh apoptosis protection.

Design and optimization of an Nrh peptide-based inhibitor

On the basis of previously demonstrated activity, we formulated a cell-permeable peptide with Nrh 1-23, grafting at its N-terminus a TAT cell penetrating domain, a short spacer, and an FITC group to allow tracking within cells. Initially, we experienced some trouble to get an efficient delivery of the TAT-Nrh 1-23 peptide in MDA-MB-231 cells. Thus, we used a light source to excite the FITC group, causing endosomal leakage and release of TAT-Nrh 1-23 into the cytosol, as previously described (Muthukrishnan et al., 2012). In the latter study however, it was shown that photochemical endosomal leakage induced apoptosis through the release of calcium from the endosomes. However, we did not report such induction of cell death in our conditions. Indeed, irradiation of the cells in presence of TAT Nrh 1-23 resulted on massive apoptosis, whereas it has no effect on cells incubated with the negative control peptide (TAT Nrh 1-23 Y16F). In fact, these latter cells behaves just like non treated cells (**See Article 2 - Figure 5**). We showed TAT Nrh 1-23 but not TAT Nrh 1-23 Y16F was able to disrupt Nrh/IP3R1 interaction *in vitro* in cell lysates, and also *in cellulo* as assessed by proximity ligation assay (PLA), the later only when photochemical endosome lysis was performed.



Figure 19 – TAT-Nrh 1-23 photochemically enhanced internalisation

In the experiment displayed above, MDA-MB-231 cells were plated on Nunc-Labtek chambered coverslides 24h prior to exposure with FITC labelled TAT-Nrh 1-23. Treatment for 4h with TAT-Nrh 1-23 in regular medium was performed, then illuminated for 2 minutes using a 488nm light source (fluorescence microscope). The illuminated spot is visible on objective x4 pictures as bright fluorescent cells, while non-illuminated cells are much less bright due to FITC quenching within endosomes. This method is therefore limited to both *ex vivo/in cellulo* applications, and has low throughput as only a small part of the sample can be readily illuminated with conventional fluorescence microscope.

Remarkably, disruption of the Nrh/IP3R1 interaction was shown to correlate with increased sensitivity to thapsigargin-induced apoptosis. In addition, we were able to monitor TAT-Nrh 1-23 effect on Nrh/IP3Rs interaction on MDA-MB-231 cells. It would be worth demonstrating that Nrh/IP3R3 is also affected, but we have not found a suitable antibody for IP3R3 detection in PLA as yet.

Even if TAT-Nrh 1-23 effect was convincing in MDA-MB-231 cells, we have been looking for a more pratical tool that would not require any external physical agents for adequate internalization. Recently, a newly discovered TAT peptide was shown to use N-terminal disulfide bond-mediated dimerization to increase its endosomal leakage capacity (Erazo-Oliveras et al., 2014). This peptide was described to act as an endosomal lytic agent that promotes cellular uptake of small molecules, proteins or even bigger cargo macromolecules present in the surrounding medium. We therefore experimented whether this strategy would work with a covalent linked cargo (Nrh 1-23 peptide), and called this new peptide dfTAT-Nrh 1-23. Indeed, we detected the disruption of the interaction in cell lysates harvested after an hour of incubation with dfTAT-Nrh without any applied external light source.



Figure 20 - dfTAT-Nrh 1-23 peptide escape endosomes without any external agent

On the top, dfTAT-Nrh 1-23 TMR labelled has the ability to form a disulfide bond and dimerize on oxidative conditions due to a residue cysteine at its N-terminus. TMR group is grafted on the side chain of lysine to minimize interference with peptide structure. On the bottom, bright fluorescence displayed by MDA-MB-231 and HS578T breast carcinoma cell lines incubated for 1h with dfTAT-Nrh 1-23 shows that the peptide has a cytosolic distribution and is not trapped in endosomes.

However, this system has some limitations, as the disulfide bond needs to be formed prior to cell treatment and maintained for the duration of the incubation with the peptide. As a consequence, a non-reducing medium must be used during the whole incubation. In our case, we used the same Balanced Salt Solution as for calcium measurements, which did not contain a potential reducer and proved to be adequate for the task. Incubation time had to be shortened to one hour as this calcium free-medium might eventually affect cell adhesion to the support, then had to be replaced with regular medium.

Oxydation of the disulfide bond prior to the use of dfTAT-Nrh 1-23 was done similarly to a previously described method (Erazo-Oliveras et al., 2014), by overnight incubation of the peptide diluted in water to 100μ M. Oxygen content of the solution has been shown to be sufficient for disulfide bond formation over a long period of time (Erazo-Oliveras et al., 2014).

We also observed that a short incubation time (90 minutes) of dfTAT-Nrh 1-23 in water with H2O2 (400µM) would work well to induce dimerization, without affecting TMR fluorescence. We did not seek to assess directly dfTAT-Nrh 1-23 dimerization in the various conditions, because we did not have readily access to a High Pressure Liquid Chromatography (HPLC) system.

Assement of dfTAT-Nrh 1-23 specifity and potency

Very interestingly, we confirmed the specifity of this peptide-based approach, as only MDA-MB-231 parental cells were sensitized to tharpsirgargin treatment, as opposed to MDA-MB-231 Nrh KO cells or MCF10A (non-transformed Nrh low cells).

Therefore, the pull-down experiments showing Nrh 1-23 specificity for Nrh and these additional data suggest that our peptide is unlikely to have an "off-target" effect.

We used two breast cancer cell lines, MDA-MB-231 and CAL51 cells to assess the potency of dfTAT-Nrh 1-23 in combination with conventional chemotherapy treatments. We observed that these two cell lines did not respond to the same drugs in the preliminary screen we made prior to our experiments, for example etoposide seemed to have a marginal effect on CAL51 cells and was not used afterwards. MDA-MB-231 responded best to dfTAT-Nrh 1-23 using etoposide and doxorubicin combination.

On the other hand, CAL51 growth was impaired by the combination of dfTAT-Nrh 1-23 with azacytidine and doxorubicin (25μ M and 0.1μ M respectively).

The last cell proliferation experiments seem to be quite interesting, because bioavailable on-site drug concentrations are much lower *in vivo* than the concentration used *in cellulo* to induce cell death, due to dose-limiting effects and pharmacokinetic drug parameters.

Still, at reduced drug concentration we have a sizable effect of dfTAT-Nrh 1-23 combination on cell growth over a short window (96h). This effect is expected to be calcium dependent, but may not necessarily involve apoptosis. Indeed, it has been demonstrated recently that excessive calcium signaling from IP3R2 is linked with enhanced ER to mitochondria calcium transfer, stimulating oxidative metabolism and ROS production. This aberrant ROS production led to induction of cell senescence and cell growth arrest (Wiel et al., 2014). Although in our case IP3R2 is clearly not involved, it can be speculated that excessive IP3R1/IP3R3 signaling resulting from Nrh inhibition may impair cell growth through a similar mechanism.

We tested this hypothesis with a longer lasting experiment, using a soft-agar colony formation assay.

We used a 1h peptide incubation followed by 24h drug pre-treatment before seeding the 2500 cells of each conditions in agar/regular medium mix, as a chronic exposure would totally impair growth. We had to decrease drug concentration from 4μ M in cell monolayer culture proliferation to 1μ M in soft agar, as the 3D growth conditions were slightly detrimental to MDA-MB-231 and found to increase etoposide toxicity. We observed that over the time course of 14 days, dfTAT-Nrh 1-23 alone did not affect significantly colony formation, but combined with 1μ M etoposide, actually reduced colony number from 93 ± 15 for control dfTAT-Nrh 1-23 Y16F peptide to a merely 28 ± 2 for dfTAT-Nrh 1-23. Finally, this result would have to be confirmed *in vivo*.

We recently engineered CRISPR CAS9-mediated Nrh KO in MDA-MB-231 cells that we put to use to verify dfTAT-Nrh 1-23 specific effect. However, we have yet to explore the many opportunities offered by such a tool. We look forward to compare parental MDA-MB-231 and MDA-MB-231 Nrh KO cell lines growth in orthotopic injection in mouse mammary gland of nude mice, followed by treatement with different doses of etoposide.

We may eventually test a formulation of Nrh 1-23 *in vivo*, but only after thoroughly testing and optimizing the numerous parameters that are critical to make such an approach viable, e.g. peptide serum stability, on-site bioavailability, oxidation/dimerization status of the peptide.

DISCUSSION

Critical assessment of BCL2L10/Nrh relevancy in human cancer

During my PhD, I took most of my time focusing on characterizing the BCL2L10/Nrh molecular mechanism that could be involved in tumour progression. We did not seek at first to verify the clinical data obtained in earlier studies (See Figure 19). This should be done in the future, to confirm the clinical relevancy of Nrh as a disease marker, hence its importance as a therapeutic target. As shown in Article 2 - Complementary Figure 1, we have set up conditions for Nrh detection by IHC in collaboration with the translational research and transfer anatomo-pathology lab at the Leon Berard Cancer Center, Lyon. Paraffin-embeded tumor samples would be stained for Nrh to demonstrate a clear link between clinical parameters (e.g. overall survival, disease-free survival...) and Nrh expression level in patient cohort for both breast and lung cancer. In fact, we have recently demonstrated that Nrh expression can be found in lung cancer cell lines and this pathology will be also considered in this study. Concerning haematopoietic diseases, it was shown in a previous study that detection of Nrh in leukemic cancer cells could be done by flow cytometry, after permeabilisation and staining of Nrh (Cluzeau et al., 2012). This method can be seen as unusual compared to a simpler quantitative PCR (qPCR), but as we have discussed in Introduction 1.6.2 & 4.1 part, BCL2L10/Nrh protein expression is regulated at posttranslationnal level by ubiquitination, therefore mRNA quantification may not be representative of actual Nrh protein level. In addition to that point, we had difficulties amplifying *nrh* gene both for molecular cloning and qPCR, due to its high GC content.

Nonetheless, we have set up experimental conditions for endogenous detection of Nrh mRNA by qPCR, and run some samples from leukemic cells, successfully detecting Nrh in Azacytidine-treated patients. More work on this direction could be done in the future, although we would focus more on breast and lung cancer for now, having better access to clinical samples in these pathologies.

On the other hand, detection of IP3R isoforms could be an interesting parameter to check. IP3 receptors have not been reported to be overexpressed in cancer cells, but the distribution of their 3 isoforms expression can vary widely across different tissues.

It has been reported for instance that some lymphoma cells expressing mainly IP3R2 relied heavily on Bcl-2 overexpression to negatively regulate IP3R2-mediated calcium signals and keep in check toxic calcium signaling (Akl et al., 2013, Ivanova et al., 2014). We characterized Nrh as primarly involved in regulating IP3R1 and IP3R3. Therefore, cartography of IP3Rs isoform expression within a tumor might give some clues on its possible dependency toward specific Bcl-2 protein (e.g. Bcl-2 or Nrh) expression to suppress potential toxic calcium signals.

We have set up the conditions for detecting IP3Rs mRNA by qPCR, but this method may be inadapted to analyze paraffin-embeded sample. Thereby, we will have to set up experimental conditions for antibody-mediated IP3Rs staining compatible with IHC.

Future development of BCL2L10/Nrh inhibitors

We have shown Nrh 1-23 peptide potency to inhibit IP3Rs/Nrh interaction by binding specifically to Nrh. Two different paths can be followed for further developpement of Nrh inhibitors: **optimization of Nrh 1-23 peptide**, or **design of a small organic molecule inhibitor** having similar functions.

First, we experienced with the Nrh 1-23 peptide that some critical points could be optimized. Our cytosolic target, Nrh, is the source of major difficulties, as we have to make the peptide membrane-permeable.

We used the TAT sequence from HIV virus, mainly because numerous experimental data are available in the literature. However, other cell penetrating sequences could be used, although not tested yet, such as penetratin or polyarginine sequence (Copolovici et al., 2014). Another interesting peptide is the pHlip peptide, which is folded in alpha helix confering cell-penetrating properties only at acidic pH. Given the fact that acidic pH is a hallmark of tumour environnement, recent studies have demonstrated that pHlip peptide can be used to specifically target tumour cells within a whole organism (Weerakkody et al., 2013). Moreover, this tool has been already used to target breast tumors (Adochite et al., 2014).

We used a 5 amino acids GGSGG spacer in our formulation of Nrh 1-23 peptide, as the highly charged TAT domain might compromise Nrh 1-23 helical structure. Therefore, linker length and properties had to be finely tuned to match the used cell penetrating domain.

For *in vivo* use of Nrh 1-23 peptide, serum stability and half-life of the peptide must be evaluated, as well as on-site biovailability that will reflect concentrations that can be reached at the tumor site in animal models. If needed, mutation may be carried out to remove potential protease cleavage site within the sequence, without impairing the peptide biological function.

To sum up, the peptidic inhibitor approach offers the advantage of high specificity and low toxicity from degradation byproducts, as amino acids would be promptly eliminated (or even reused!) by the human body. These advantages come with a tradeoff on stability and membrane penetration of the peptide.

To circumvent these limitations, small organic molecules may offer better stability and membrane penetration, but may also jeopardize specificity and degradation byproducts may have a higher toxicity compared to an optimized Nrh 1-23.

In order to find a novel Nrh inhibitor, we plan to set up an *in vitro* readout allowing for high-throuput screening of molecules binding Nrh and disrupting Nrh/IP3R1 interaction. We are currently producing a sizable quantity of recombinant Nrh protein, envisioning at first to study Nrh 1-23 binding on full-length Nrh, to gain access for Nrh 1-23 affinity constant for Nrh.

We may then perform the screen using small molecules to interfere with Nrh 1-23/full-length Nrh interaction, to single out hits that will affect Nrh 1-23. Indeed, screening of molecules binding Bcl-2 BH4 domain has been shown to give the succesfull lead compound BDA-366 (Han et al., 2015). We hypothesize that an *in silico* screen could be also performed and give interesting results, as Nrh structure has been determined by X-ray crystallography (PDB n°: 4B4S).

Finally, peptidic and small organic molecule approaches offer complementary solutions that could be both investigated. Only rigorous *in vivo* testing should be able to discriminate the best method to target Nrh for efficient therapeutic applications.

Perspectives for anti-Bcl-2 cancer therapeutic strategies

As we have seen in Table 3, most therapeutic strategies devised to inhibit pro-survival Bcl-2 proteins have been focused on the BH3 domain. BIRD-2 peptide has been developed to inhibit Bcl-2 BH4 domain, more precisely counter its ability to negatively regulate calcium fluxes and apoptosis through the interaction with IP3Rs. BDA-366 compound has also been developed as a Bcl-2 BH4 antagonist, although its mechanism of action is more likely to reside in Bcl-2 conversion into a BH3-only pro-apoptotic protein.

More work is to be expected in that direction, as therapeutic targeting of Bcl-2 proteins BH4 domain would rather be more specific than targeting the common BH3 binding feature of many Bcl-2 family members. Moreover, from our own experimental data and knowledge on Bcl-2 family, each pro-survival proteins BH4 domain seems to have quite specialized and divergent functions. Recent work with BIRD-2 peptide highlighted that its combination with the best known Bcl-2 BH3 pocket inhibitor ABT-199 is a highly potent association that should be very effective to kill tumor cells, and would be a promising combination to test in clinical trials (Lavik et al., 2015; Greenberg et al., 2015). It is thought that such a combination strategy could **maximize treatment efficiency**, hence limiting possible *de novo* or acquired resistance to therapy.

Targeting BH4 domain of Bcl-2 proteins anti-apoptotic functions outside canonical BH3 binding could also help to **prevent side effect associated** with such strategies. For instance, Bcl-xL BH3 pocket pharmacological inhibitor ABT-737 has been reported to induce severe thrombocytopenia. Design of specific inhibitors for Bcl-xL BH4 domain functional interaction at the mitochondria with VDAC channels could be worth considering (**See Figure 16**).

Finally, our own work through Nrh comes also to support this idea that targeting BH4 domain of prosurvival Bcl-2 proteins should be the next step precluding novel anti-Bcl-2 strategies.

List of Abbreviations

Å: Ångström ADP: Adenosine biphosphate AIF: Apoptotic-Inducing Factor ALV: Avian Leukosis Virus AML: Acute Myeloid Leukemia AMP: Adenosine MonoPhosphate AMPK: Adenosine MonoPhosphate Kinase Apaf-1: Apoptotic protease-activating factor-1 ARM: Armadillo Repeat Fold ATG: AuTophagy-related Genes ATP: Adenosine TriPhosphate Bcl-2: B-Cell Lymphoma 2 BD: IP3R1 IP3 Binding Domain BECN1: Beclin-1 BH: Bcl-2 Homology Domain BIRD-2: Bcl-2 IP3 Receptor Disruptor-2 BSS: Balanced Salt Solution CAMKII-B: CAlModuline-dependent Kinase Kinase-beta CARD: CAspase Recruitment Domain CD: IP3R1 Coupling Domain CDK: Cyclin-Dependent Kinase CFD: Channel Forming Domain CICR: Calcium-Induced Calcium Release CK: Casein Kinase CLL: Chronic Lymphocytic Leukemia CTD: C-Terminal Domain Da: Dalton DAG: DiAcylGlycerol DD: Death Domain DED: Death Effector Domain DFCP1: Double FYVE-Containing Protein 1 DL-BCL: Diffuse Large B-Cell Lymphoma DNA: DeoxyriboNucleic Acid EM: Electron Microscopy ER: Endoplasmic Reticulum

ERK1/2: Extracellular signal-Regulated Kinase FADD: Fas-Associated Death Domain FITC: Fluorescein IsoThioCyanate GSK-3: Glycogen Synthase Kinase GTP: Guanosine Tri-phosphate HD: Helical Domain HIV: Human Immunodeficiency Virus IAP: Inhibitor of Apoptosis Proteins ICE: Interleukin-1-β-Converting Enzyme IKK: IkB Kinase IL-3: Interleukin 3 ILD: Intervening Lateral Domain IMM: Inner Mitochondrial Membrane IMS: InterMembrane Space IP3: Inositol 1,4,5-triphosphate IP3R: Inositol 1,4,5-triphosphate Receptor JNK: c-Jun N-terminal Kinase K_d: Affinity Constant KO: Knock-Out LNK: LiNKer domain MAM: Mitochondria-Associated Membranes MAPK: Mitogen-Activated Protein Kinases MCU: Mitochondrial Calcium Uniporter MDS: MyeloDysplatic Syndroms MEF: Mouse Embryonic Fibroblast MFN2: Mitofusin-2 miRNA: microRNA MLCK: Myosin Light Chain Kinase MO: Morpholino MOMP: Mitochondrial Outer Membrane Permeabilization mPTP: mitochondrial Permeability Transition Pore mRNA: messenger RNA MSK1: Mitogen- and Stress-activated Kinase 1 MTD: IP3R1 Modulatory and Transducing Domain mTOR: mammalian TOR mTORC1: mTOR Complex 1 NMR: Nuclear Magnetic Resonance

NO: Nitric Oxyde OMM: Outer Mitochondrial Membrane PAS: Phagophore Assembly Site PCR : Polymerase Chain Reaction PEST: Proline, Glutamate, Serine Threonine rich domain PI3K: PhosphatidylInositol-3-Kinase PIP₂: Phosphatidylinositol-biphosphate PIP₃: Phosphatidylinositol-triphosphate PKA: Protein Kinase A PKC: Protein Kinase C PLA: Proximity Ligation Assay PLC: PhosphoLipase C RAPTOR: Regulatory-Associated Partner of mTOR RNA: RiboNucleic Acid **ROS:** Reactive Oxygen Species **RSV: Rous Sarcoma Virus** RyR: Ryanodyne Receptor qPCR: quantitative Polymerase Chain Reaction SAHB: Stabilized Alpha-Helix of Bcl-2 domain SAR: Structure-Affinity Relationship SCLC: Small-Cell Lung Cancer SD: IP3R1 Suppressor Domain siRNA: short interfering RNA TCA: Tricarboxylic Acid Cycle TCR: T-Cell Receptor TM: TransMembrane TMD: TransMembrane Domain TMR: TetraMethylRhodamine TNF: Tumor Necrosis Factor TNFR: TNF Receptor TOR: Target Of Rapamycin TRAF6: TNFR-Associated Factor 6 UPR: Unfolded Protein Response VDAC: Voltage-Dependent Anion Channel WIPI: WD-40 repeats Interacting with Phosphatidyl Inositol YSL: Yolk Syncytial Layer β-TRF: β-Trefoil Fold

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