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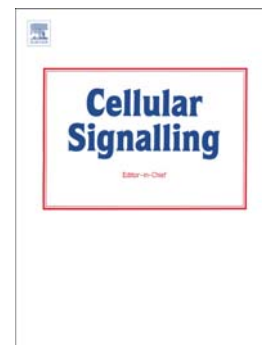
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The phosphorylation of Metaxin 1 controls Bak activation during TNF α induced cell death

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Abbreviations. AMOMP: apoptotic mitochondrial outer membrane permeability; BdGBM: Bax deficient Glioblastoma Multiforme; CHX: cycloheximide; IP: Immunoprecipitation; IVT: in vitro translated; Mtx: metaxin; MOM: mitochondrial outer membrane; PP1: PP1-analog II 1NM-PP1; VDAC: Voltage dependent anionic channel; TNF: tumor necrosis factor; TOM: translocase of outer membrane.

Summary

The proapoptotic protein Bak is implicated in the execution phase of apoptosis, a cell death programme. Bak is essentially mitochondrial and during early steps of apoptosis undergoes conformational changes that lead to its full membrane integration in mitochondria and the subsequent liberation of pro-apoptotic mitochondrial proteins. Little is known about the partners and mechanisms implicated in the activation of Bak. We have recently shown that Bak is incorporated into a Voltage dependent Anionic Channel of type 2 (VDAC2)/Metaxin 1(Mtx1)/Metaxin 2 (Mtx2) multi-protein complex in both resting and dying cells. Here, we show that, after the induction of apoptosis, Bak switches from its association with Mtx2 and VDAC2 to a closer association with Mtx1. This change of partners is under the control of a tyrosine phosphorylation of Mtx1 by c-Abl.

1. Introduction

Apoptosis is a cell death program, which is central to the maintenance of cellular and tissue homeostasis as well as normal differentiation and ageing. Abnormal regulation of apoptosis is involved in major pathologies both through its escalation (e.g. in neurodegenerative disorders and autoimmune diseases) or its inhibition (e.g. cancers) (1,2). Bak and Bax are pro-apoptotic members of the BCL-2 family that are essential in the initiation of apoptosis (3). During the early steps of apoptosis, Bak undergoes several changes of conformation to induce modifications of mitochondrial permeability associated with the liberation of pro-apoptotic mitochondrial proteins (3). Activation of Bak is under the control of several stimuli including the so-called BH3 only proteins, a class of BCL-2 like proteins (3) but also mitochondrial proteins like VDAC2 and Metaxin1 and 2 (4-6). Bak is maintained in an inactive state by its association with the voltage dependent anionic channel type-2 (VDAC2), an isoform of the mitochondrial porins (VDACs) (7,8). However, the exact role of VDAC2 is not fully understood, as it has also been shown to promote apoptosis by recruiting newly synthesized Bak to mitochondria (9).

Metaxin 1 and Metaxin 2 (Mtx1 and Mtx2) are peripheral membrane proteins involved in nuclear-encoded protein import into mitochondria (10-12). Metaxins have been identified more recently as members of the Sorting and Assembly Machinery (SAM) (13,14) which facilitate the membrane insertion of beta-barrel proteins family which include VDAC (Höhr *et al.*, 2015). Mtx1 has been shown to be required for the TNF-induced cell death (15) and that its deficiency led to mitochondrial anti-apoptotic phenotypes (16). Recently, we have shown that Bak interacted with Mtx2 in resting cells and with Mtx1 upon TNF α treatment (6)

The role and the regulation of apoptosis by the different mitochondrial receptors of the Bcl-2 family are not completely understood and it has been speculated that it could affect cell death only under specific physiological situations (17, 18). In this study, we show that the

interaction of Bak with Metaxin 1 upon induction of apoptosis is under the control of Mtx1 phosphorylation. This interaction could constitute an essential step for death receptor -induced apoptosis.

2. Materials & Methods

2.1 materials

Unless indicated all reagents used in this study are from Sigma-Aldrich.

2.2 Statistical analysis.

All experiments were done at least in triplicates. Significance of the differences in means were calculated using Student-t-test, while correlations were determined using Pearson-test.

2.3 Proximity ligation In Situ Assay (P-LISA)

Cells were fixed with 4% paraformaldehyde in PBS pH7.4 for 15 min at room temperature. Permeabilization is performed with PBS containing 0.5% Triton X100 for 20 min at room temperature and staining were realized according to manufacturer's instructions (Olink Bioscience). Fluorescence was visualized by using the Axiovert 200M microscopy system (Zeiss, Le Pecq, France) with ApoTome module (x63 and numerical aperture 1.4). Acquisition was done in structured illumination microscopy as described earlier (19, 20).

2.4 Immunoprecipitation

Immunoprecipitation of Bak from mitochondria was performed using the Catch and Release v2.0 reversible immunoprecipitation system (Millipore) under non-denaturing conditions. A mixture of 2 µg anti-Bak (clone: TC-98, immunogen BH1 domain, Calbiochem) and 2 µg anti-Bak (immunogen amino acids 14-36, Pharmingen) was used. Immunoprecipitated proteins were analyzed by Western blot or by gel staining after removal of IgG using the ProteoSeek Antibody-Based Albumin/IgG Removal Kit (Pierce), according to the manufacturer's instructions. The re-probing of Western blots was performed using a ReBlot

Western blot recycling kit (Millipore) and protein detection was performed with a Visualizer Western blot detection kit (Millipore). Gels stain with SYPRO-Ruby was performed overnight according to the manufacturer's recommendations (Invitrogen). The antibodies used and their concentrations are listed in Table 1. Cellular extracts from HeLa cells were used as a control for each antibodies.

2.5 Confocal experiments and cell free assay

Acellular binding of IVT proteins to mitochondria and competition experiments, cell fractionation and confocal microscopy are identical to those described previously (Cartron *et al.*, 2003). To visualize apoptosis in BdGBM cells, we used anti-ACTIVE® caspase3 antibody (Promega #G74481) and Dapi to label nuclei as reported previously (6).

2.6 Kinase/Phosphatase activities

Abl kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂, 1.2 mM DTT, 100 μM ATP, 100 μM Signal Transduction Protein (Tyr160) biotinylated peptide (Cell Signal#1366) and 250 ng indicated recombinant protein (Interchim). Mtx1 was phosphorylated in similar conditions. Phosphatase activities were measured from biotinylated peptide previously phosphorylated using 350 ng recombinant c-Ab (Abcam #ab196111).

2.7 shRNA treatments

Three siRNAs directed against human Mtx1 were designed initially using the *siRNA target finder* web-based software from Ambion (http://www.ambion.com/techlib/misc/siRNA_design.html). The target sequences found for Mtx1, based on the sequences identified by the Ambion web-tool, were 5'-AAGATCAGCAACCCCTGGCAG-3', 5'-AATGCTGATTATGATCTTGTC-3' and 5'-AAGTGGTATGCAGAGGCTATG-3'. The oligonucleotides were annealed and cloned into the BamHI-HindIII sites of the pSilencerTM 3.1-H1 hygro siRNA expression vector (Ambion).

These vectors (10 µg) as well as a scrambled control were transfected into Du145 cells and HeLa cells by electroporation (Gene Pulser II, Biorad) using the following parameters: 250V, 250 µF for Du145 cells and 230V, 1000 µF for HeLa cells. Cells were selected in medium containing hygromycin (250 µg/mL). For c-Abl down regulation, we used SilenciX® stable silenced (knockdown) HeLa cells and a control SilenciX® cells (transfected with a non-relevant shRNA sequence) as instructed by the manufacturer (TEBU-France).

2.8 Site directed mutagenesis

Plasmids carrying site-directed mutants (Y228N, Y232N and Y268N) of Mtx1 were created using the GeneTailor kit (Invitrogen), and transfected to previously obtained shMTX1 cells. The identity of all constructs was verified by sequence analysis.

3. Results

3.1 Role of Mtx1 and Mtx2 in Bak association with mitochondria.

We have recently shown that Metaxin 1 and 2 interacted sequentially with Bak upon induction of apoptosis by a TNFα/ cycloheximide (TNFα/CHX) treatment (6). We studied the effect of the knock down of the expression of Mtx1 and Mtx2 by interference RNA (shRNA) as previously described (6) on the subcellular localization of Bak in resting and TNFα/ CHX treated primary cultures from Bax deficient *Glioblastoma multiforme* patients (BdGM) (6). We used as a control two sh Scrambled and sh TOM22, directed against another protein import receptor which has been involved in Bax docking to mitochondria (21) and the analysis of Bcl-2 subcellular localization as it is a protein closely related to Bak (3). As shown in **Figure 1A**, confocal analyses using Mitotracker to label live mitochondria reveal that MTX2 knock down decrease the co-localisation of Bak with mitochondria in resting but not in apoptotic cells. Conversely, MTX1 knock down did not affect Bak localization in resting cells but decreases its association during apoptosis. On the other hand, neither the

treatment by sh scrambled nor shTOM22 affected Bak localization. Of note, Bcl-2 mitochondrial association was not affected by neither of these treatments. We used cellular fractionation as described earlier (21) to further assess Bak subcellular localization. **Figure 1B** illustrates an immunoblot analysis of two fractions: the cytosolic and the heavy membranes (which contains mitochondria) compartments. We confirmed the effect of the differential knock down of MTX2 and MTX1 on Bak mitochondrial localization but we also observe that the cytosolic re-localization of unbound Bak into the cytosol when MTX2 was inhibited in resting cells and in apoptotic cells when MTX1 was absent. To investigate the effect of a cytosolic component in this re-localization, we used mitochondrial fraction (HMF) in an acellular assay to study the association of an in vitro translated Bak (IVT-Bak) (Cartron *et al.*, 2014). As illustrated in **Figure 1C**, Bak was not associated to MTX2-deficient HMF and a mild trypsin treatment designed to proteolyze exposed domains of Bak (6) indicated that bound Bak was essentially peripheral in control or MTX1-deficient HMF. We treated IVT-Bak by a pro-apoptotic truncated form of Bid (p15-tBid) and MTX1 deficient HMF could not support Bak association to membrane. Of note, under these conditions p15-tBid treated IVT-Bak was protected from trypsin in control and MTX2-deficient HMF, indicating a possible membrane insertion of Bak under these conditions (**Figure 1C**).

3.2 Mtx1 phosphorylation controls its interaction with Bak.

We investigated the putative role of posttranslational modifications upon TNF α /CHX induced apoptosis by analyzing the phosphorylation status of Bak and MTX1. Mitochondrial outer membrane from resting or apoptotic cells primary cultures from BdGM were first immunoprecipitated by anti Bak, anti MTX1 or anti MTX2 antibodies as described earlier (6). The immunoprecipitated fractions were then analyzed by immunoblots with antiphosphotyrosine,

antiphosphoserine and antiphosphothreonine antibodies to determine both the nature of the phosphorylation and to estimate the amount of phosphorylated proteins. As illustrated in **figure 2A**, in resting BdGBM cells, Mtx1, but not Bak or Mtx2, was tyrosine phosphorylated (pTyr-Mtx1) and no other type of phosphorylation was detected. Of note, we observed a significant decrease in pTyr-Mtx1 during TNF α /CHX induced apoptosis and no modification for MTX2 and Bak (**Figure 1A**).

BdGBM treated with 1nM PP1-analog II (1NM-PP1), a cell permeable broad kinase inhibitor, exhibited a significant decrease in pTyr-Mtx1 both in resting and in TNF α /CHX-treated cells (**Figure 2B**). In BdGBM, treated with 1 mM of a tyrosine phosphatase inhibitor S-orthovanadate (S-ortho), the decrease pTyr-Mtx1 during apoptosis was abolished (**Figure 2B**). In parallel, we estimated the level of apoptosis in these cells by measuring the DEVDase activity. As illustrated in **Figure 2C**, we noted that 1NM-PP1 treatment at 1nM shortened the onset of DEVDase maximal activity, while S-ortho treatment decreased the DEVDase activity drastically. We used a proximity ligand assay (see materials and methods) to quantify the interaction between Bak and Mtx1, under resting and apoptotic conditions using VDAC2 as a control. Interestingly, after 12h, Proximity ligation assays showed that 1NM-PP1 treatment increased the number of Bak/Mtx1 interactions and decreased the number of Bak/VDAC2 interactions in comparison to resting cells (**Figure 2D**). Conversely, S-ortho treatment limited the number of Bak/Mtx1 interactions and increased the Bak/VDAC2 interactions in comparison to untreated cells (**Figure 2D**). Taken together these results suggest that 1NM-PP1 treatment acts as an “enhancer” of the TNF α /CHX-induced Bak-mediated apoptosis by promoting Bak/Mtx1 interactions and decreasing Bak/VDAC2 interactions, while S-ortho inhibits Bak-mediated apoptosis by promoting the Bak/VDAC2 interactions and decreasing Bak/Mtx1 interactions.

3.3 Mtx1 phosphorylation may be mediated by the c-Abl/Ship2 couple.

Putative phosphorylation sites are present in the human MTX1 sequence (**Figure S1**). We determine the capacity of selected recombinant tyrosine kinases to promote the phosphorylation of Mtx1 in an acellular system in which the kinase dependent ^{32}P -incorporation into IVT-Mtx1 was quantified. Among the panel of five tyrosine kinases included, only c-Abl supported the ^{32}P -incorporation into IVT-Mtx1 (**Figure 3A**). To substantiate this observation, we analyzed the tyrosine phosphorylation of IVT-Mtx1 by cytosols obtained from cells treated or not with 1NM-PP1 and/or immuno-depleted in c-Abl. The depletion of Scr, the major target of 1NM-PP1, was used as control. Western blot analyses showed that the IVT-pTyr-Mtx1 was markedly decreased in the presence of cytosols treated with 1NM-PP1 or immunodepleted in c-Abl, while Scr depletion did not affect IVT-pTyr-Mtx1 (**Figure 3B**). PPT1 has been shown to moderately inhibit c-Abl (Liu *et al.*, 1999). To validate the role of c-Abl in Mtx1 phosphorylation, we confirmed that the kinase activity of c-Abl decreased in cytosol immunodepleted in c-Abl and in cytosols obtained from cells treated with 1NM-PP1 (**Figure 3C**).

To identify the phosphatase implicated into the dephosphorylation of Mtx1, we initially used apoptotic cytosols from cell treated with S-ortho or Na-stibogluconate. Different doses of phosphatase inhibitors were used in order to inhibit Ship1 (15 μM) or Ship2 (120 μM). Western blots analyses revealed that the de-phosphorylation of Mtx1 was observed when IVT-Mtx1 was incubated with cytosol obtained from cells treated with 120 μM Na-stibogluconate (**Figure 3D**). This suggests that Ship2 is the phosphatase implicated into the Mtx1 dephosphorylation. This was confirmed by the observation that incubation of IVT-Mtx1 with Ship2-immunodepleted apoptotic cytosol did not modify pTyr-Mtx1 levels (**Figure 3D**). The involvement of Ship2 in the dephosphorylation of Mtx1 was strengthened by the fact that apoptotic cytosol phosphatase activity was affected by both 15 μM Na-stibogluconate and

Ship2 immunodepletion (**Figure 3E**).

3.4 Role of phosphorylation in the interaction Mtx1/ Bak

To confirm that the phosphorylation status of Mtx1 promote its interaction with Bak, we performed a two-step acellular interaction assay. Firstly, the acellular tyrosine phosphorylation of IVT-Mtx1 was efficiently achieved by its incubation with recombinant c-Abl (**Figure 4A**). Phosphorylated and non-phosphorylated Mtx1 were incubated with p15-tBid activated Bak before immunoprecipitation with anti-Mtx1 antibodies. As shown in **Figure 4A**, Bak was not associated with phosphorylated Mtx1. This result suggests that phosphorylation of Mtx1 is determinant for its interaction with Bak. Analysis of potential tyrosine phosphorylation site in Mtx1 indicated 3 potential sites for c-Abl (**Figure S1**). We mutated the three putative tyrosine phosphorylation sites for an asparagine and, analyzed ^{32}P -incorporation into IVT-Mtx1 into the mutants described in **Figure 3A**. **Figure 4B** shows that only the mutation of Y228 exhibited a reduced incorporation of ^{32}P . To verify if the mutations affected the acellular binding of Bak to Mtx1, we performed an acellular assay similar to that described in **Figure 4A** with the different mutants and analyzed the presence of Bak co-immunoprecipitated with anti-Mtx1. As shown in **Figure 4C**, the binding of Bak to Mtx1 was not affected by the addition of c-Abl only in the Mtx1Y228N mutant, suggesting that this residue was important for the regulation of the interaction. To analyze the impact in vitro of Y mutations we used an indirect method. As previously described (6), the C-terminal transmembrane truncated Mtx1 (Mtx1DN) is a dominant negative inhibitor of TNF α /CHX induced apoptosis. We used Mtx1DN constructs with Y228N, Y232N, or Y268N mutations and analyze their influence on apoptosis induced by TNF α /CHX in BdGBM cells. We postulated that non phosphorylated Mtx1DN would be a better inhibitor of apoptosis because of an increase in its binding to Bak. As shown in **Figure 4D**, Y228N mutation enhanced

apoptosis protection by Mtx1DN while Y232N has a less important effect and Y268N no effect on apoptosis. These results are in good agreement with the acellular results and point out Y228 (and possibly Y232) as phosphorylated residues implicated in Bak's binding during apoptosis. However since the mutation of Y228 did not completely abolish the P32 labelling (**Figure 4B**) it is likely that other phosphorylations could be involved in the process as suggested by the existence of multiple phosphorylation sites in this MTX1 region (**Figure S1B**).

3.5 C-Abl inhibition enhanced the tumor-necrosis-factor related apoptosis inducing ligand (TRAIL) dependent apoptosis through the increase of Mtx1/Bak interaction.

The persistent inhibition of c-Abl has been shown to enhance TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) induced apoptosis (22). Since TRAIL and TNF α are related, we postulated that the Bak /Mtx1 interaction would also be involved in trail induced cell death. We thus tested the effect of Mtx1 phosphorylation on TRAIL induced apoptosis in HeLa cells. As shown in **Figure 5A**, inhibition of by STI-571 induced a moderate apoptosis which was inhibited by the down regulation of Mtx1. Next, we analyze the co-localization between Bak and Mtx1 in cells treated either with Si c-Abl or Si scramble as a control. As illustrated in **Figures 5B 5C**, the co-localisation of the two proteins was not affected in control cells. However, the knock down of the expression of c-Abl reduced the co-localization between Bak and Mtx1 in untreated cells while the induction of apoptosis by TRAIL enhanced this co-localization. It is noteworthy that the downregulation of c-Abl appears to reduce the amount of Bak bound to mitochondria per itself while that of Mtx1 has no influence on Bak mitochondrial localization in resting cells (**Figure 1A**). A similar situation was found in TNF α /CHX treated HeLa cells (**data not shown**).

4. Discussion

In recent years, several mechanisms have been suggested to account for apoptotic mitochondrial outer membrane permeability (AMOMP) (1-3). In most cases only some lipids, some members of the Bcl-2 family and VDAC2 have been implicated in this process (7). VDAC2 has been shown to directly interact with inactive Bak and specific structural changes are required to displace Bak to another complex which allows its oligomerization and insertion into the mitochondrial outer membrane during apoptosis (5, 9).

VDAC2 is also implicated in several mitochondrial functions, beyond its anti-apoptotic role, including steroidogenesis, both activities possibly regulated by its phosphorylation (23).

Quite interestingly, recent publications have implicated in several members of the Bcl-2 functions the proteins of the machinery for nuclear-encoded protein import machineries TOM and SAM (6, 17, 21).

Wang *et al.* (24) have shown that Mtx1 was required for TNF α /CHX, UV or γ -irradiation-induced cell death, but was not necessary for Fas-induced apoptosis. We have recently identified Mtx2 and 1 as receptors for Bak during TNF α /CHX induced apoptosis (6). Quite interestingly, recent results have shown that TOM machinery is essential in the mitochondrial elimination during autophagy by controlling the PINK-PARK pathway (25, 26). These results confirm that mitochondrial protein import machineries are implicated in cell survival not only through regulation of metabolism but also through the control of cell death programs. However, modulators of these functions remained unknown.

The major finding of our work is that a decrease in the phosphorylation of Mtx1 occurs during apoptosis and is associated with Bak change of conformation. Acellular experiments further indicate that the dephosphorylation is sufficient to enhance the binding of Mtx1 to Bak and that one kinase, c-Abl, and one phosphatase, Ship2, are putative candidate. The role of c-Abl was further established by both pharmacological inhibition and knock down of its expression.

c-Abl is localized in the nuclei and in the cytoplasm (**Figure S2**) and a dual role in apoptosis has been proposed (26) in which nuclear c-Abl is pro-apoptotic whereas cytoplasmic c-Abl could actively inhibit apoptosis upon specific stimuli.

Taken together, our data suggest the model depicted in **Figure 6**. In non-apoptotic cells, Bak is docked on the mitochondrial membrane by its interactions with VDAC2 and Mtx2, the COOH-terminus playing an important role at this stage. Bak might also associate with the anti-apoptotic proteins Mcl-1, and possibly Bcl-xL. Upon TNF α /CHX-induced apoptosis, Bak can be released from Mtx2/VDAC2 to interact with Mtx1. Our results suggest that the latter stage could be under the control of both a change of conformation in Bak and Mtx1 phosphorylation. The Bak/Mtx1 interaction is under the control of specific tyrosine phosphorylations (i.e. Y228 and possibly Y232) resulting from the combined activity of the kinase c-Abl and its cognate phosphatase Ship2. This dephosphorylation of Mtx1 promotes the association with Bak and its pro-apoptotic insertion in the outer membrane. However, the impact of c-Abl regulated Bak activity is difficult to establish as this kinase has been shown to be involved in different steps of its activation including through the protein kinase C delta-p38 MAPK signaling or Bcl-xL up-regulation (28). Recently, tyrosine phosphorylation of Bak has been shown to control its activation during apoptosis (29, 30). Strikingly, our data indicate that the tyrosine dephosphorylation of Mtx1 is required for the insertion of Bak into MOM during apoptosis. Since we did not observe, under our conditions, any phosphorylation of Bak, it would be interesting to know if both mechanisms are mutually exclusive or if they can be concomitant.

The importance of phosphorylation as a key mechanism in the control of mitochondrial physiology including the functions of several Bcl-2 family proteins (31) and in protein translocation into yeast mitochondria (32) has been recently documented. Interestingly, several kinases have been implicated at different levels of the protein import; for example

casein kinase 2 controls the assembly of TOM complex while protein kinase A controls its function and connects protein import to major signaling pathways (32).

5. Conclusion

This study not only provides a new mechanistic view into Bak-induced apoptosis but also identifies new and important partners in the complex process of AMOM. Of note, Mtx1 has been implicated in the targeting, membrane insertion and function of two major proteins of MOM: the cholesterol-binding translocator proteins (33) and VDAC (34). It remains to evaluate if the Mtx 1 phosphorylation by c-Abl are universally involved in all types of Bak-dependent apoptosis and the molecular mechanism by which they assist Bak integration in MOM.

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Conflict of interest

There are no conflicting interests.

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Legends of figures

Figure 1: role of Mtx1 and MTx2 in Bak subcellular localization in human glioma Bax-deficient cell line.

A) Confocal microscopy analysis of Bak and Bcl-2 subcellular localization after TNF/CHX treatment of BdGBM (see materials and methods), Bak and Bcl-2 were visualized by specific antibodies and a mitochondrial probe (MitoTracker green). In parallel, these analyses were performed in cells treated with shTOM22 or shMTX1 or shMTX2 and control (shScr) (see materials and methods). CF= correlation factor between a mitochondrial probe (MitoTracker green) and Bak or Bcl-2, 100 cells were used in each experiment. Bak is cytosolic in siMtx2 treated cells, but not in wild type, siTOM22 treated BdGBM cells. Note that the subcellular localization of Bcl-2 was not affected by any of these treatments. Illustrations are representative of at least 5 different experiments. **B)** BdGBM cells, transfected with siMtx1 or siMtx2 were subjected to cell fractionation as described in materials and methods. In healthy and in TNF/CHX treated cells, the presence of Bak in cytosolic and mitochondrial fractions was analyzed by immunoblots using F1-ATPase as a mitochondrial marker and actin as a cytosolic marker. **C)** Heavy membrane fractions obtained from shMtx1 or shMtx2 treated BdGBM were used in an acellular assay in which Bak association to mitochondria upon activation by p15-tBid was assessed as described in Cartron et al.,(6). The results shown are the mean of 4 independent experiments.

Figure 2: decrease of Mtx1 phosphorylation during apoptosis. **A)** top: Bak was immunoprecipitated from resting or UV-treated HeLa and analyzed by immunoblots with anti-Bak antibodies, anti phosphotyrosine (pTyr), phosphoserine (pSer), phosphothreonine (pThr); Mtx1 and Mtx2 phosphorylation were analyzed similarly in apoptotic and resting cells after immunoprecipitation with anti-Mtx1 or Mtx2 antibodies and anti-pTyr, anti-pSer and anti-pThr. **B)** Control and apoptotic HeLa were treated with PP1 analog II (1 nM) or Sodium

Orthovanadate (S. ortho) (1mM) and Mtx1 phosphorylation probed with anti-pTyr. **C)** HeLa cells were exposed to PP1 analog II or Sodium Orthovanadate (S. ortho) and UV treated. Cell death as monitored by measuring caspase 3 (i.e. DEVDase) activity and **D)** Protein-protein interactions were determined by PLA as described in Materials and methods using Bak/ Mtx1 or Bak/VDAC2 as coupled antibodies. 200 cells were counted for each condition.

Figure 3: role of Ship2 and c-Abl in the phosphorylation of Mtx1. **A)** To determine which tyrosine kinase promoted the phosphorylation of Mtx1, we measured the ^{32}P -incorporation into in vitro translated Mtx1 protein (IVT-Mtx1) upon incubation with recombinant Jak2, Syk, Fym, c-Abl and c-Src. **B)** IVT-Mtx1 was incubated with control cytosol treated or not with PP1 or immunodepleted in c-Abl (Abl1 dep) or Src (Src dep). Top: fluorography of Mtx1 and bottom : western blot with anti phosphotyrosine antibodies. **C)** In parallel, the abl kinase activity was measured in the treated or untreated cytosol was measured as described in materials and methods. **D)** IVT-Mtx1 was incubated with apoptotic cytosol treated or not with sodium orthovanadate (S. ortho), sodium stibogluconate (S. Stibo.) at two concentrations with distinct inhibitor properties (see text) or immunodepleted in Ship2 (Ship2 dep.), top: fluorography of Mtx1 and its phosphorylation viewed by Western blot with anti-phosphotyrosine antibodies. **E)** Phosphatase activities were determined in the different apoptotic cytosolic fractions in which phosphatase inhibitor was added or depleted with Ship2 antibody.

Figure 4: effect of phosphorylation on the interaction between Bak and Mtx1 **A)** IVT-Mtx1 was incubated in the presence of recombinant c-Abl: top: fluorography of Mtx1 and its phosphorylation viewed by Western blot with anti-phosphotyrosine antibodies. P15-treated Bak (as in Figure 1C) was incubated or in the presence of Mtx1 treated or not with c-Abl and the mixture was immunoprecipitated with anti-Bak antibodies (IP Bak) or anti-Mtx1

antibodies (IP Mtx1). The presence of Bak and Mtx1 was analyzed after stripping and reprobing of the Western blots. Data illustrated in this figure are representative of at least 3 independent experiments. **B)** Site directed mutagenesis was used to substitute asparagine to tyrosine at position 228, 232 and 268. The different mutants were incubated upon in vitro translocation in the presence or in the absence of recombinant c-Abl and the incorporation of ^{32}P was used to monitor phosphorylation. **C)** The different IVT Bak mutants were incubated in the presence of c-Abl and Mtx1 before immunoprecipitation with anti Bak antibodies. The presence of co immuno-precipitated Bak and Mtx1 was assessed by immunoblots. **D)** C-terminally truncated Mtx1 mutants were overexpressed in BdGBM and its effect on TNF α /CHX induced cell death was assessed as a percentage of control (i.e. no constructs added) by trypan blue staining.

Figure 5: impact of Mtx1 on c-Abl -dependent apoptosis

A) SilenciX® stable HeLa cell line silenced for c-Abl were treated by STI-571 (μM) in the presence or not of siMtx1. Cell death was assessed by trypan blue and expressed as percentage of dead cells **B)** Confocal microscopy analyses of TRAIL induced apoptosis in SilenciX® stable HeLa cell line silenced for c-Abl. The co-localization of Mtx1 (red) and Bak (green) is shown in the pictures and **C)** quantification of the correlation factors (CF) is represented by an histogram .

Figure 6: model of Bak activation upon induction of apoptosis. In resting cells Bak is associated either with mitochondrial anti apoptotic proteins of the Bcl-2 family such as Mcl-1 (and possibly Bcl-xL) or to VDAC2 and Mtx2. Upon induction of apoptosis (i.e. tBid) Bak is transferred totally or partially to Mtx1 and then is integrated into the outer membrane of mitochondria. The association of Bak with Mtx1 is prevented by the phosphorylation of the latter protein, which is under the control of c-Abl and Ship2. This phosphorylation is decreased during apoptosis enabling thus Mtx1 to interact with Bak.

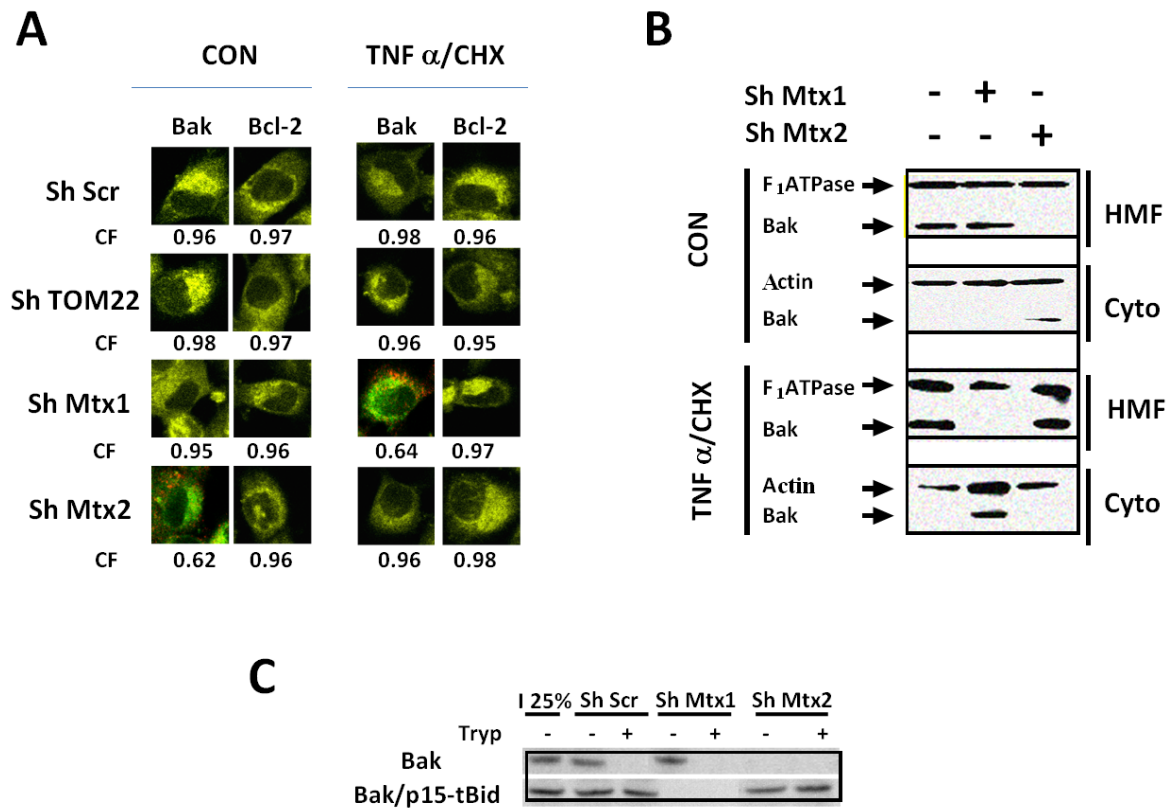


Figure 1

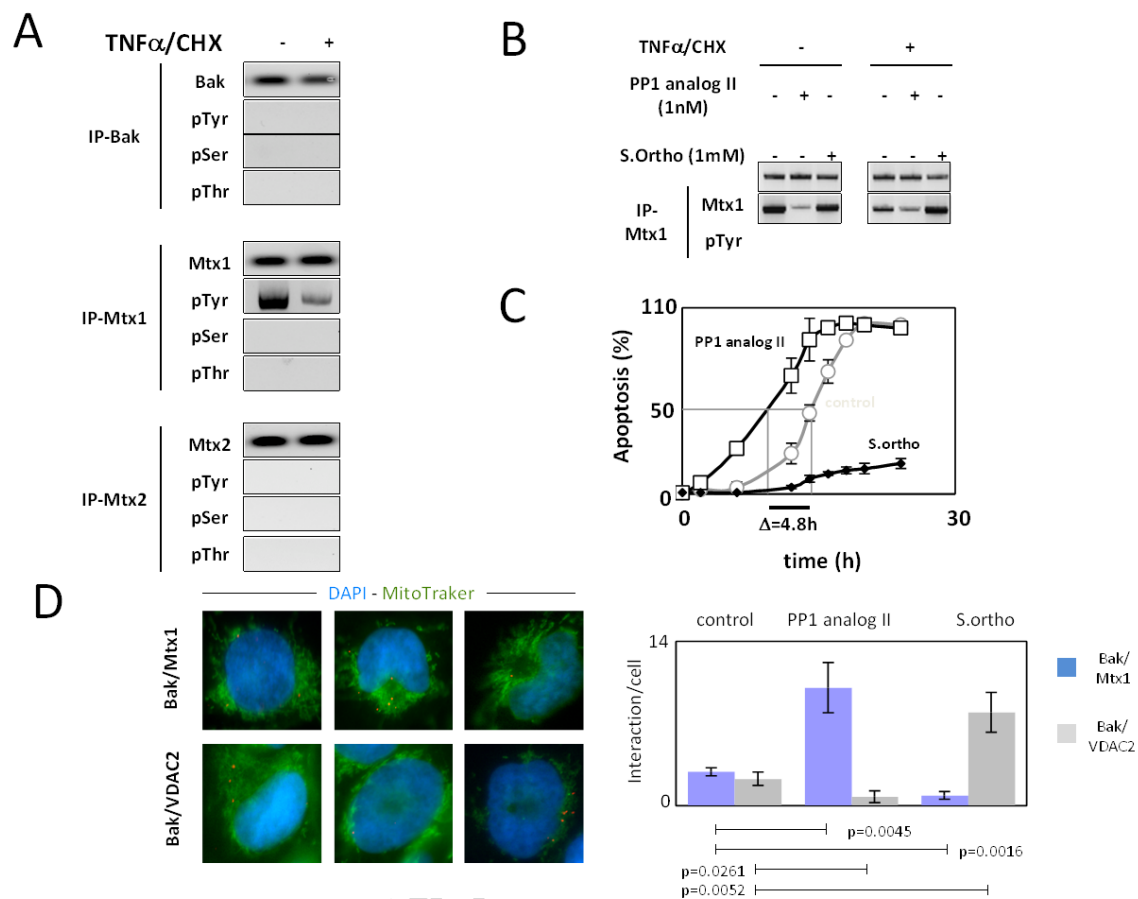


Figure 2

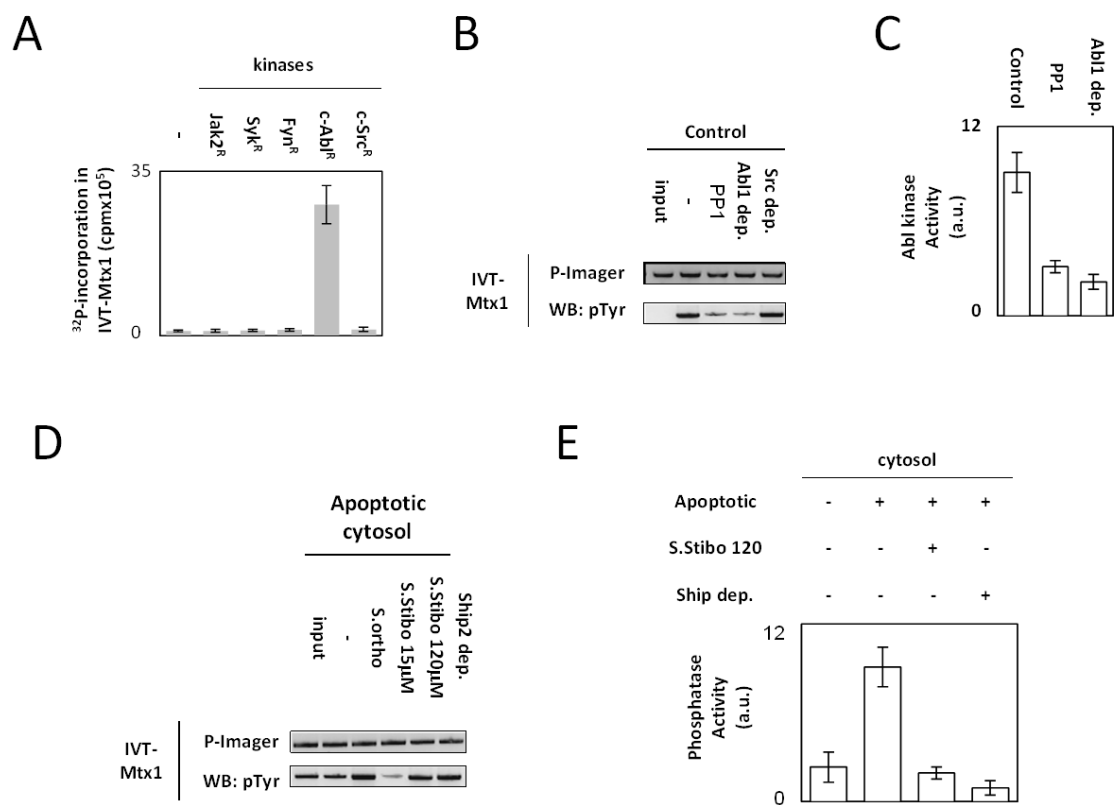


Figure 3

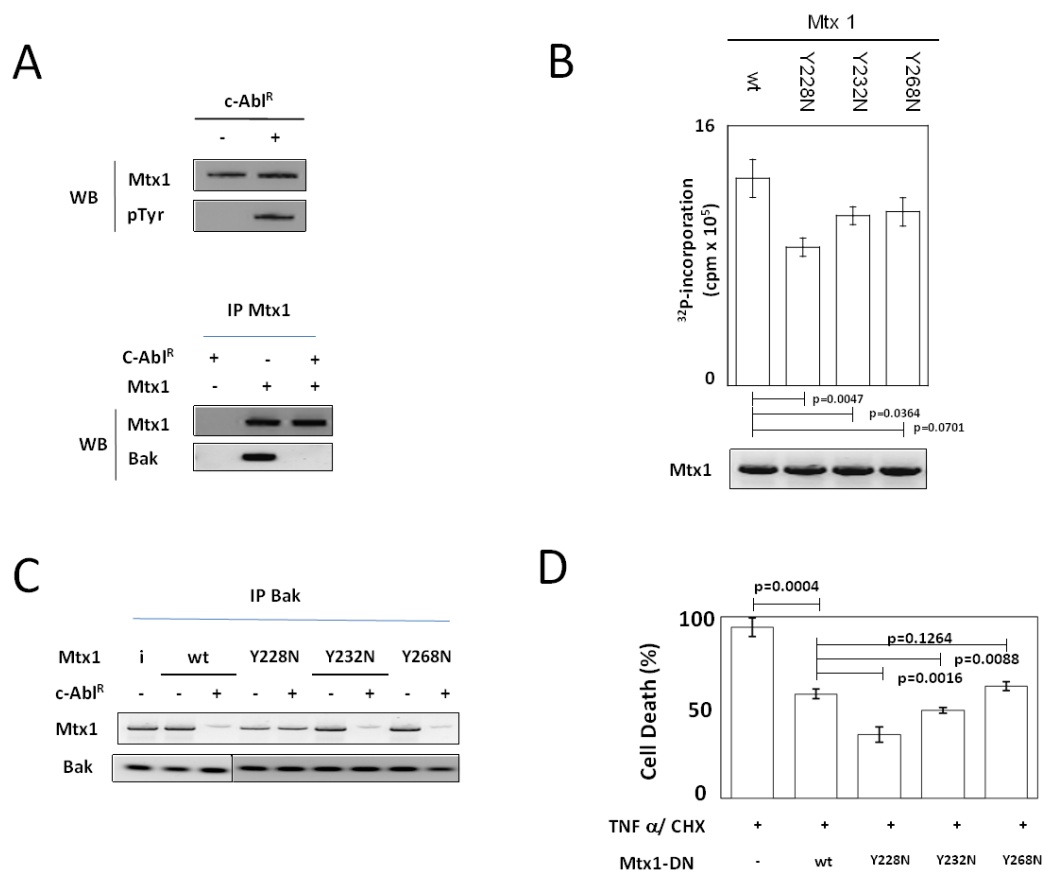


Figure 4

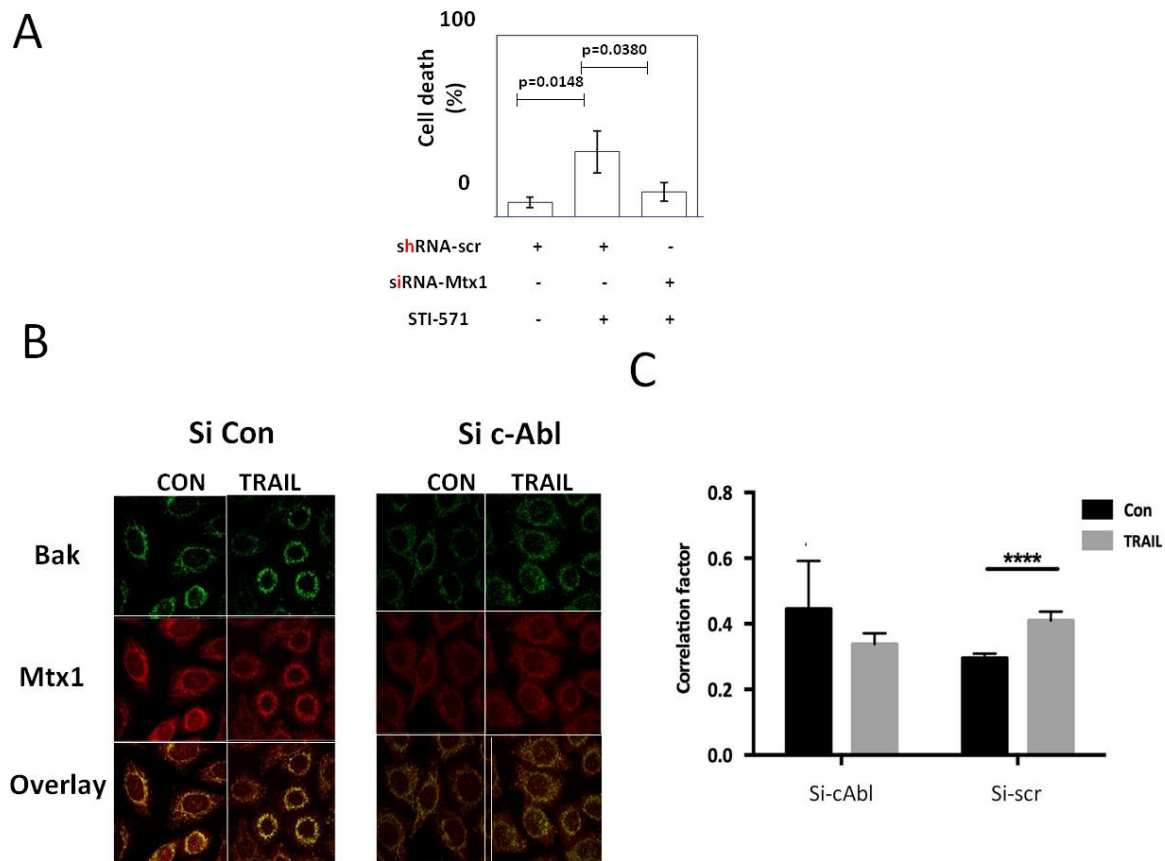


Figure 5

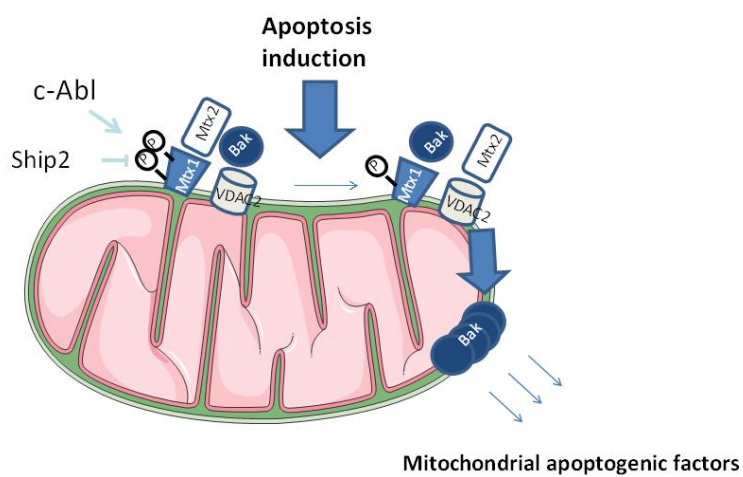
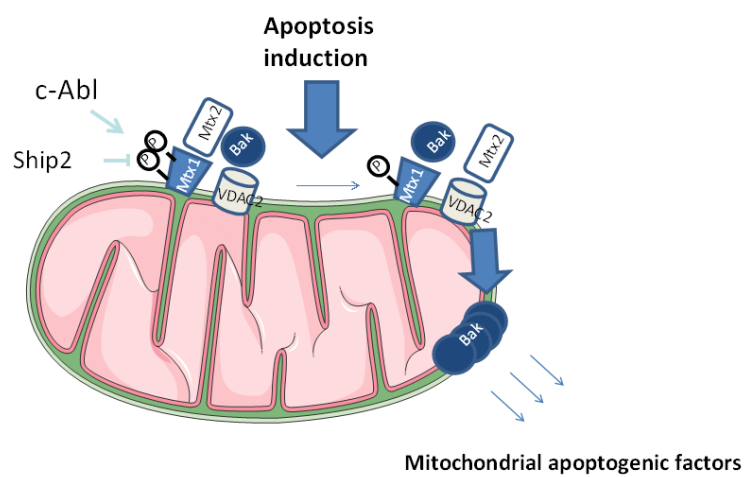


Figure 6

Graphical abstract : Petit et al.



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Highlights

1. Bak, a proapoptotic member of the Bcl-2 family, associates with Metaxin 1, a member of the sorting and assembly machinery of the outer membrane of mitochondria, upon TNF alpha induced cell death
2. a decrease of the phosphorylation of MTX1 is linked to the association of Bak with Metaxin 1
3. c-abl and ship2 are likely to be implicated in the phosphorylation/ dephosphorylation of MTX1.