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**Mitochondrial involvement in  
cell death: release of pro-  
apoptotic proteins**

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## Abstract

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In addition to the role of the mitochondria in energy metabolism, these organelles play a key role in cell death signaling. In particular, mitochondrial alterations, such as stimulation of reactive oxygen species (ROS) formation, decreased ATP synthesis, loss of membrane potential and the release of pro-apoptotic proteins from the mitochondrial intermembrane space (IMS), have been shown to be involved in, and often responsible for, various manifestations of cell death. Among the pro-apoptotic proteins to be released are cytochrome *c* and Apoptosis-Inducing Factor (AIF). While cytochrome *c* initiates caspase-dependent cell death, AIF leads to caspase-independent cell death. The release of pro-apoptotic proteins from the IMS is considered as a “point of no return” in apoptotic signaling, although the mechanisms regulating permeabilization of the outer mitochondrial membrane remain controversial. Two main mechanisms have been elaborated including the induction of mitochondrial permeability transition (MPT) and selective pore formation by the Bcl-2 (B-cell lymphoma 2) family proteins, Bax and Bak. Although, distinct cell death pathways can be triggered by various signals, they often merge at a common regulator of this multistep process. Currently, it is widely accepted that the mitochondria serve as such a regulator. Therefore, the main goal of this project was to investigate the involvement of mitochondria in cell death.

While cytosolic Bax is known to target the mitochondria to induce mitochondrial outer membrane permeabilization (MOMP) during apoptosis, less is known about its mitochondria-specific target. We addressed this issue and could show that a functional translocase of the outer mitochondrial membrane (TOM complex) is required for Bax to release cytochrome *c* from the mitochondria.

Previous work in our group has established that AIF-mediated, and caspase-independent apoptosis is the main cell death mechanism in non-small cell lung carcinomas treated with anticancer drugs. However, the molecular mechanisms underlying AIF release remained obscure. We addressed this issue and could show that  $\text{Ca}^{2+}$  was imported into cells upon treatment of cells with protein kinase C (PKC) inhibitors. The imported  $\text{Ca}^{2+}$  had two critical functions; a) the activation of a mitochondrial calpain that could cleave AIF, a step essential for its release and b) to stimulate mitochondrial ROS production. This, in turn, led to a selective posttranslational modification, carbonylation of AIF, which significantly sensitized AIF to calpain-mediated processing. Recently, we were also able to define the pathway by which  $\text{Ca}^{2+}$  entered cells exposed to the PKC inhibitors. We found that the hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) mediated a sustained  $\text{Ca}^{2+}$  import, which was triggered by dephosphorylation of a critical residue within the conserved C-terminus of the channel in cells treated with PKC-inhibitors. Our findings reveal a novel role for the HCN2 channel in cell death signaling and uncover a new potential therapeutic drug target.

## List of Publications

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- I. Ott M, **Norberg E**, Walter KM, Schreiner P, Kemper C, Rapaport D, Zhivotovsky B, Orrenius S. The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome c release.  
*J Biol Chem.* 2007; 282,27633-27639.
- II. **Norberg E**, Gogvadze V, Ott M, Horn M, Uhlén P, Orrenius S, Zhivotovsky B. An increase in intracellular Ca<sup>2+</sup> is required for the activation of mitochondrial calpain to release AIF during cell death.  
*Cell Death Differ.* 2008; 15,1857-1864.
- III. **Norberg E**, Gogvadze V, Vakifahmetoglu H, Orrenius S, Zhivotovsky B. Oxidative modification sensitizes mitochondrial apoptosis-inducing factor to calpain-mediated processing.  
*Free Radic Biol Med.* 2010; 48,791-797.
- IV. **Norberg E**, Karlsson M, Korenovska O, Szydlowski S, Silberberg G, Uhlén P, Orrenius S, Zhivotovsky B. Critical role for hyperpolarization-activated cyclic nucleotide-gated channel 2 in the AIF-mediated apoptosis.  
*EMBO J.* 2010; 29,3869-3878.

### Additional publications (not included in the thesis)

- I. Ott M\*, **Norberg E\***, Zhivotovsky B, Orrenius S. Mitochondrial targeting of tBid/Bax: a role for the TOM complex?  
*Cell Death Differ.* 2009; 16,1075-1082.  
\* Denotes equal contribution
- II. Gogvadze V, **Norberg E**, Orrenius S, Zhivotovsky B. Involvement of Ca<sup>2+</sup> and ROS in alpha-tocopheryl succinate-induced mitochondrial permeabilization.  
*Int J Cancer.* 2010; 127,1823-1832.
- III. **Norberg E**, Orrenius S, Zhivotovsky B. Mitochondrial regulation of cell death: processing of apoptosis-inducing factor (AIF).  
*Biochem Biophys Res Commun.* 2010; 396,95-100.

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## List of abbreviations

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AIF	Apoptosis-Inducing Factor
ANT	Adenine nucleotide translocase
Apaf-1	Apoptotic protease activating factor 1
ATP	Adenosine tri-phosphate
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl	B cell lymphoma
Bcl-X <sub>L</sub>	Bcl-2 related gene, long isoform
BH	Bcl-2 homology
Bid	BH3-interacting-domain death agonist
Bim	Bcl-2 interacting mediator of cell death
Calpain	Calcium-activated neutral protease
CARD	Caspase requirement domain
Caspase	Cysteine-dependent aspartate-specific protease
DIABLO	Direct IAP-binding protein with low pI
DISC	Death-inducing signaling complex
EndoG	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
HtrA2	High temperature requirement protein A2
IAP	Inhibitor of Apoptosis protein
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
Omi	Omi stress-regulated endoprotease
OMM	Outer mitochondrial membrane
MOMP	Mitochondrial outermembrane permeabilization
MLS	Mitochondrial localization signal
NLS	Nuclear localization signal
PARP	Poly (ADP-ribose) polymerase
Puma	P53-upregulated modulator of apoptosis
PKC412	N-Benzoyl staurosporine
ROS	Reactive oxygen species
Smac	Second mitochondrial-derived activator of caspases/DIABLO
STS	Staurosporine
tBid	Truncated Bid
TNF	Tumor necrosis factor
TOM	Translocase of outer membrane
TRADD	TNF-receptor-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
XIAP	X chromosome-linked IAP
VDAC	Voltage-dependent anion channel



## General introduction

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Deletion of cells was long thought to occur spontaneously in a passive mode, until 1972, when Kerr, Wyllie and Currie discovered a gene-regulated form of cell death, which they called apoptosis<sup>1</sup>. The authors demonstrated that normal cell turnover as well as spontaneous elimination of potentially harmful cells, such as cancer cells, occurred by this mechanism. In addition, it was shown that apoptosis can be triggered or inhibited by a variety of physiological and pathological stimuli. Apoptosis was compared to pathological necrosis, which represents a more passive way of eliminating cells. While necrosis leads to the burst of the plasma membrane followed by the leakage of the intracellular content into the extracellular milieu, recruiting an immune response, the apoptotic cells remain intact and are rapidly engulfed by macrophages.

Today, it is known that cell populations are highly regulated by proliferation, differentiation and death. Dysregulation of any one of these three processes might lead to uncontrolled growth or uncontrolled death of a cell. Insufficient or excessive apoptosis has been implicated in various disorders. For instance, cancer and rheumatoid arthritis are diseases with inadequate amount of apoptosis, while neurodegeneration, as in Parkinson's disease or Alzheimer's disease, is associated with excessive apoptosis<sup>2</sup>. In 2002, Sydney Brenner, John Sulston and Robert Horvitz were awarded with the Nobel Prize in Physiology or Medicine for their discovery of genes involved in programmed cell death and the characterization of their precise function during development of the nematode, *Caenorhabditis elegans*.

During recent years it has become clear that several distinct modes of cell death exist and the Nomenclature Committee on Cell Death in 2009 proposed to distinguish 12 modes of cell death<sup>3</sup>.

### Mechanisms of apoptosis

Apoptosis is by far the most studied mode of cell death to date. It represents a highly regulated process requiring ATP, as well as RNA and protein synthesis<sup>4</sup>. Apoptosis can be triggered by either intracellular or by extracellular stimuli. There are several morphological features characterizing apoptosis, including membrane blebbing, reduction of cellular volume, chromatin condensation and fragmentation of the nuclei. All these events contribute to the final stage of apoptosis, namely the formation of apoptotic bodies. Apoptotic cells exhibit "eat me" signals and are rapidly "eaten" or phagocytosed, by neighboring cells or professional macrophages<sup>5</sup>. Multiple biochemical features are associated with apoptosis, the most important being cleavage of cellular proteins by a family of proteolytic enzymes, the caspases, which cleave hundreds of substrates<sup>6</sup>. Other features include the permeabilization of the outer mitochondrial membrane, leading to the release of pro-apoptotic proteins into the cytosol<sup>7</sup>, activation of DNases that degrade the DNA and the exposure of an "eat me" signal, phosphatidylserine, on the plasma membrane<sup>8</sup>.

## Extrinsic and intrinsic apoptotic pathways

As mentioned earlier, apoptosis can be triggered by different stimuli, originating intracellularly or extracellularly. Different types of stimuli activate various signaling pathways of apoptosis. The pathway to be activated depends also on the cell type and its genetic signature as well. The two most well characterized pathways are the extrinsic (receptor-mediated)<sup>9</sup> and the intrinsic (mitochondria-mediated)<sup>10</sup> pathways. The extrinsic pathway is initiated when a death ligand binds its receptor on the cell surface. The intrinsic pathway, on the other hand, is activated by a signal that arises inside the cell and proceeds via the mitochondrial release of pro-apoptotic proteins. Despite different stimuli, the extrinsic and the intrinsic pathways both result in the same morphological features, defining an apoptotic cell.

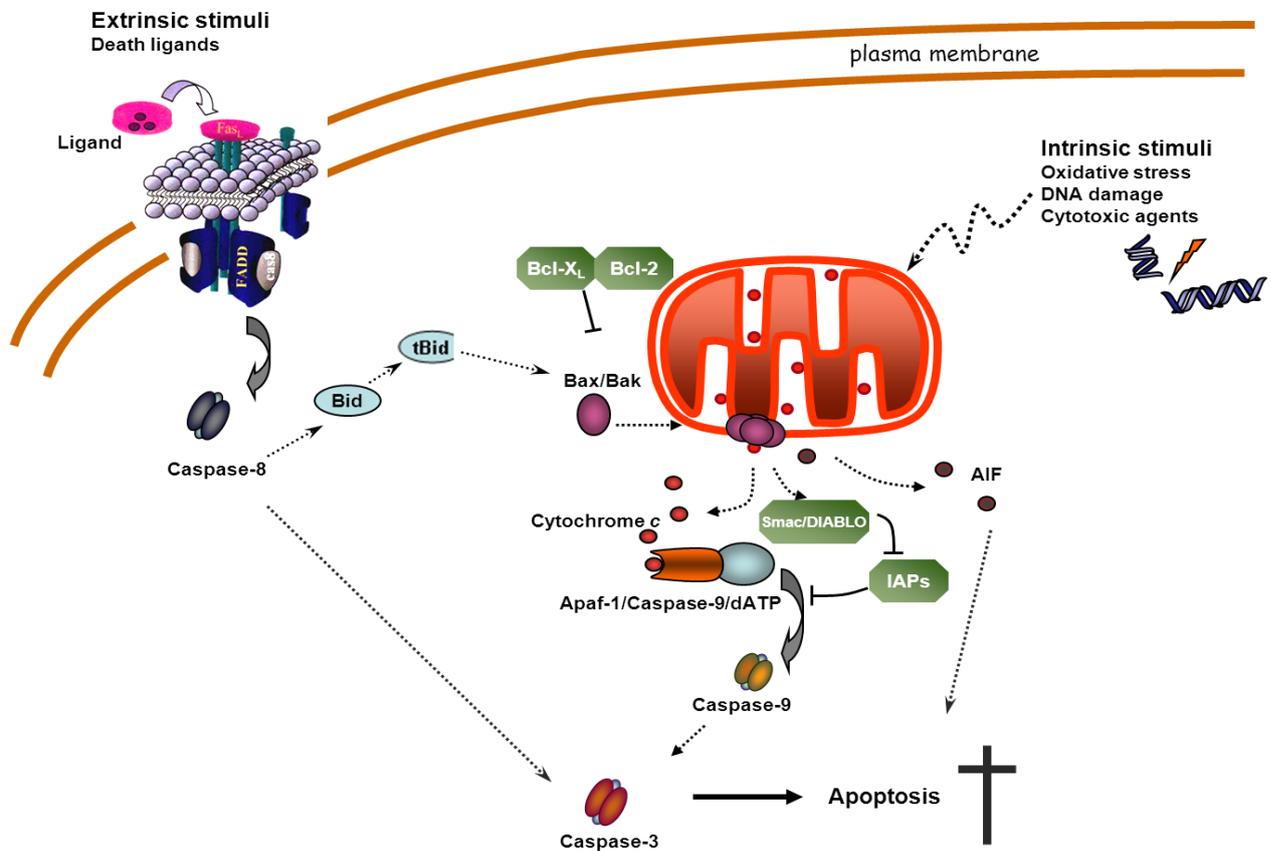
The death receptors, belonging to the tumor necrosis factor (TNF) superfamily, on the plasma membrane are important for the extrinsic pathway. The main death receptors are CD95 (Apo-1/Fas), TNF receptor 1A (TNF-R1), and the TNF-related apoptosis-inducing ligand receptors (TRAIL) DR4 and DR5<sup>11,12</sup>.

The CD95 and TNF receptors consist of a receptor exposed on the cell surface, transmembrane regions and an intracellular death domain. Binding of a ligand to the corresponding receptor will cause aggregation of the receptors, which transduces the extracellular stimuli to a signal inside the cell. The aggregation of death domains of three receptors leads to the recruitment of adaptor proteins, called FADD or TRADD, and pro-caspase-8/-10. Altogether, these proteins form the death inducing signaling complex (DISC) which ultimately leads to the activation of initiator caspase-8/-10. These, in turn, cleave and activate effector caspase-3, -6 and -7, which can further cleave and degrade proteins culminating in apoptosis<sup>13</sup>.

Beyond its well established role in energy metabolism, mitochondria are key participants in the cell death machinery<sup>14,15</sup>. In particular, the mitochondrial outer membrane permeabilization (MOMP) is considered as an important event for cell death. Once the pro-apoptotic proteins that normally reside in the intermembrane space (IMS) of mitochondria are being released into the cytosol, they will initiate apoptosis<sup>16,17</sup>. Some of the mitochondrial proteins that are being released are: Apoptosis-Inducing Factor, cytochrome *c*, Endonuclease G (EndoG), second mitochondrial activator of caspases (Smac)/direct IAP binding protein with low pI (DIABLO), and Omi, stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2)<sup>18</sup>. Several theories have been elaborated to describe how these proteins can be liberated from the mitochondria, including selective disruption of the outer mitochondrial membrane (OMM) as a result of swelling of the mitochondrial matrix<sup>19,20</sup> and pore formation mediated by members of the Bcl-2 family of proteins, notably Bax and Bak<sup>21</sup>.

The most extensively studied protein to be released is cytochrome *c* that normally is a component of the electron transport chain (ETC). Once in the cytosol, it interacts with Apaf-1 and pro-caspase-9 in the presence of dATP to form the apoptosome complex, which is essential for the activation of caspase-9. Activated caspase-9 can then cleave and activate effector caspase-3 and -7. The effector caspases subsequently cleave their substrates, leading to apoptosis<sup>18,22</sup>. AIF and EndoG are the key molecules of caspase-independent cell death. They both translocate from the mitochondria, via the cytosol to the nucleus, where they are involved in chromatin condensation and DNA fragmentation<sup>16</sup>.

There is a crosstalk between the extrinsic and the intrinsic pathways. This is mainly mediated by caspase-8 that can cleave Bid into tBid, which can promote MOMP and the subsequent mitochondria-mediated apoptosis<sup>23</sup>. In addition, caspase-9 can cleave and activate caspase-3, which in turn can activate caspase-8, leading to the amplification of the initial apoptotic stimuli.



**Figure 1. Overview of the extrinsic and the intrinsic pathways of apoptosis.**

### The Bcl-2 family of proteins

The Bcl-2 family and related proteins include more than 30 proteins which can be divided into three subgroups: Bcl-2-like survival factors, Bax-like death factors and BH3-only death factors. These can either promote (Bax, Bak, Bad, Puma, Noxa, Bid, Bcl-X<sub>s</sub>) or inhibit (Bcl-2, Bcl-X<sub>L</sub>, Bcl-w, Mcl-1, A1/Bfl-1) apoptosis<sup>24,25</sup>. The Bcl-2 family proteins contain BH1-4 domains. Pro-survival (or anti-apoptotic) proteins have at least BH1, BH2 and BH4 and a transmembrane domain (TM) in the C-terminus, allowing membrane insertion. The anti-apoptotic Bcl-2 family proteins have been reported to be located throughout the cell including in the membranes of endoplasmic reticulum (ER), OMM, nuclei, and lysosomes<sup>26</sup>.

The prime function in apoptosis of all of Bcl-2 family proteins is to either promote or to prevent MOMP. Therefore, the balance between pro-apoptotic and anti-apoptotic proteins determines whether a cell undergoes apoptosis or not, according to the rheostat model<sup>27</sup>.

Pro-apoptotic Bax and Bak normally exist as inactive monomers and can be activated by BH3-only proteins, like Bid. Activation of Bax and Bak includes conformational changes within the protein structure. These re-arrangements are essential for their oligomerization and pore formation. While Bax is normally located in the cytosol, upon activation it changes conformation and translocates to the OMM to form pores. Bak, on the other hand, is already located at the OMM and, only needs the conformational change for its pro-apoptotic function<sup>28</sup>. The precise role of BH3-only proteins in activating Bax or Bak is not yet fully understood.

At present, two models exist to explain the role of BH3-only proteins in promoting apoptosis. The first, called the direct activation model, is based on binding of BH3-only proteins to Bax and Bak, leading to their activation and oligomerization<sup>29</sup>. The second, called the neutralization model, is a refined interpretation of the rheostat model. In this model, BH3-only proteins bind and neutralize the anti-apoptotic proteins, like Bcl-2 and Bcl-X<sub>L</sub>. The anti-apoptotic proteins are pro-survival proteins that normally inhibit the activation of Bax/Bak and thereby protect the mitochondrial integrity. Hence, it is possible for the Bax/Bak to insert/oligomerize and form pores in the OMM only when all the anti-apoptotic molecules are first inhibited by the BH3-only proteins<sup>30</sup>. The BH3-only proteins are in turn activated either by transcription, post-translational modifications or enzymatic truncations<sup>26</sup>.

## Caspases

Caspases are cysteine aspartate proteases that are activated during the most of apoptotic pathways and are considered as the main executioners of apoptosis as they are involved both in the early and late stages of this process. Caspases are expressed as inactive pro-enzymes in cells. Like the Bcl-2 proteins, these proteins also undergo conformational changes in order to become active. The active enzyme consists of two small and two large subunits with a critical cysteine residue in the active site<sup>31</sup>. To date, 14 caspases have been discovered in mammals, yet not all are involved in apoptosis<sup>32</sup>. For instance, Caspase-1, -4, -5, -11 and -12 are called the pro-inflammatory caspases and their primary function is the activation/maturation of cytokines. The apoptotic caspases can be divided into initiator and effector caspases. To the former group belongs caspase-2, -8, -9 and -10 and to the latter group caspase-3, -6 and -7<sup>6</sup>. Caspases are typically located in the cytosol but have been reported to also localize to the Golgi, ER, and nucleus. While not all caspases have a distinct role in apoptosis, overexpression of any one of them potentially induces cell death. More than 400 caspase substrates have been identified<sup>33</sup>.

## Caspase-dependent vs. caspase-independent apoptosis

Apoptosis is dependent, in most cases, on caspase activation and the cleavage of their substrates leads to cell death. Despite this, apoptosis has been observed in cells where caspase activity was inhibited.

The first protein that was discovered to mediate caspase-independent cell death was AIF. It was shown that addition of recombinant AIF to isolated nuclei, led to the appearance of apoptotic nuclei, even in the presence of the pan-caspase inhibitor, z-VAD-fmk<sup>34</sup>. AIF is known to translocate from the mitochondria to the nucleus during apoptosis and to contribute to chromatin condensation and large scale DNA fragmentation. While caspase-dependent cell death results in oligonucleosomal DNA fragmentation, AIF-mediated apoptosis causes peripheral chromatin condensation<sup>35</sup>.

However, the precise role of AIF in the nucleus is still unclear although this phenomenon was discovered more than a decade ago. The protein itself does not exert endonuclease activity<sup>36</sup>; however, it was suggested to act in combination with an endonuclease, EndoG<sup>37</sup>. Yet, the precise mechanism of this co-operation remains elusive.

### **Additional apoptotic regulators**

Another family of serine proteases involved in apoptosis is the granzymes which have similar substrate specificity as caspases. Granzyme A induces caspase-independent cell death by targeting a complex on the ER, which evokes the activation of nuclease during CTL-mediated killing. Granzyme B can trigger both caspase-dependent and caspase-independent modes of cell death<sup>38,39</sup>.

As mentioned earlier, MOMP constitutes one of the major checkpoint(s) of apoptotic cell death. More recently, the permeabilization of yet another organelle, the lysosome, has been shown to initiate a cell death process, however, only under specific circumstances. Lysosomal membrane permeabilization (LMP) causes the release of lysosomal peptidases, cathepsins, into the cytosol. Like the caspases, cathepsins are synthesized as inactive pro-enzymes, but are subsequently activated within the low pH environment of the lysosomes. LMP is, in particular, induced by lysosomotropic agents, such as *O*-methylserine dodecylamide hydrochloride, detergent-like sphingosine, or Leu-Leu-Ome and the antibiotic ciprofloxacin<sup>40</sup>. LMP is a potentially lethal event since the ectopic presence of lysosomal proteases in the cytosol causes digestion of vital proteins and the activation of caspases. The role of lysosomes and cathepsins in apoptosis is still a matter of debate. However, lysosomes are essential for a survival mechanism called autophagy<sup>41</sup>.

Calpains, calcium-activated cysteine proteases, are activated when the intracellular  $\text{Ca}^{2+}$  level is increased and are present as two main isoforms, calpain-I ( $\mu$ -calpain) and calpain-II (m-calpain). They normally interact with the endogenous calpain-specific inhibitor, calpastatin. This calpastatin-interaction prevents both the activation and catalytic activity of calpains. Calpain-I and -II diverge in the amount of  $\text{Ca}^{2+}$  ( $\mu\text{M}$  and  $\text{mM}$ , respectively) required for their activation. When activated, calpains can cleave and destroy their endogenous inhibitor, calpastatin. Calpastatin can also be cleaved by caspases, which thereby facilitate calpain activation. This represents yet another example of crosstalk between different pathways of apoptosis. In addition, calpains and caspases have several shared substrates including  $\text{Ca}^{2+}$ -dependent kinase, fodrin and PARP. The  $\text{Ca}^{2+}$  elevation required for the activation of calpains can originate from either import of extracellular  $\text{Ca}^{2+}$ , or from release from intracellular stores like the ER or mitochondria. Calpains are mainly cytosolic enzymes, although calpain-I has been shown to harbor a mitochondrial localization signal (MLS)<sup>42-44</sup>.

## Introduction to the present study

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### Mechanisms of mitochondrial outer membrane permeabilization

Mitochondrial permeabilization leading to the release of pro-apoptotic proteins from the IMS of mitochondria is a critical event in most of cell death pathways, and often considered as the “point of no return”. The caspase-dependent pathway can be triggered by the release of cytochrome *c*, whereas caspase-independent apoptosis results primarily from the processing and release of AIF. Two main mechanisms leading to mitochondrial permeabilization have been elaborated: induction of mitochondrial permeability transition (MPT) and Bax/Bak-mediated pore formation.

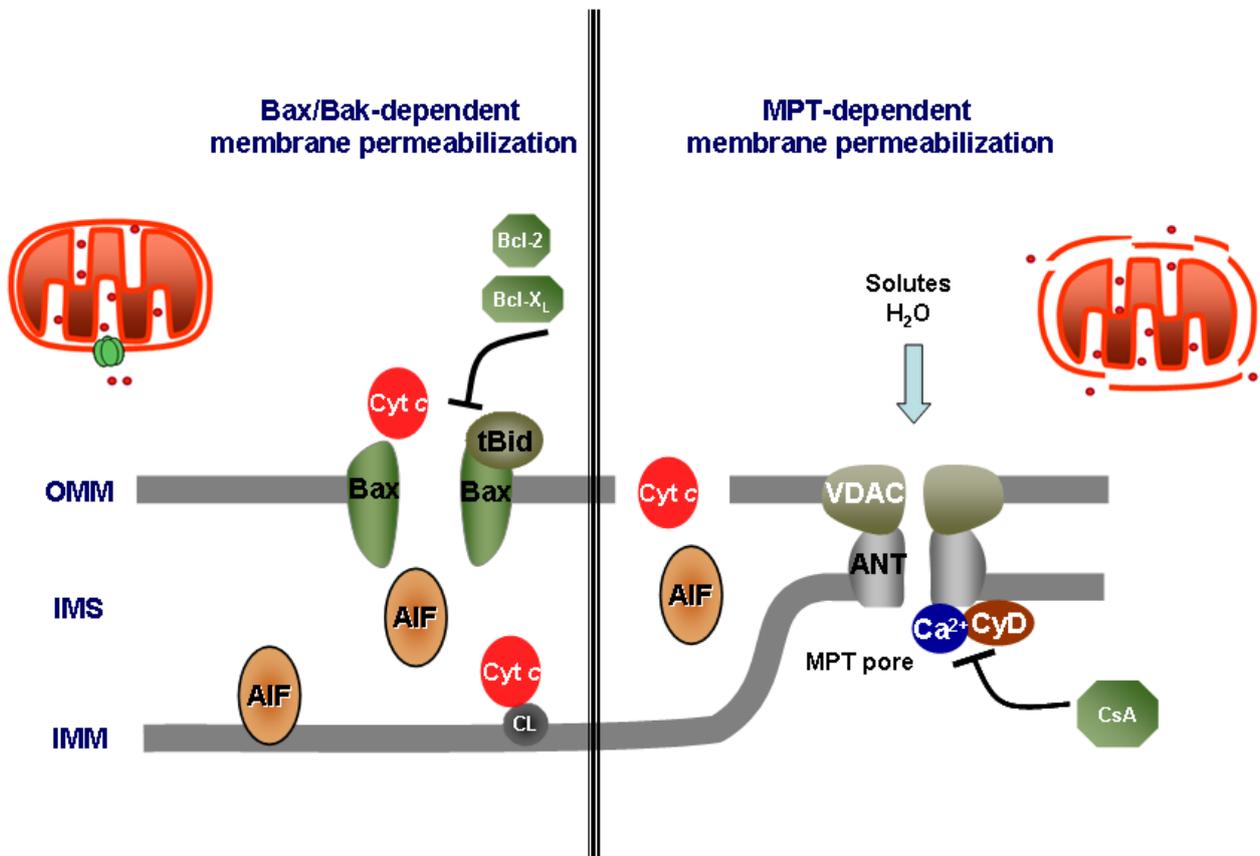
MPT was described about 30 years ago by Haworth and Hunter when they showed that mitochondrial  $\text{Ca}^{2+}$  uptake can stimulate drastic changes in the morphology of mitochondria as well as the functional activity. This was found to be due to the opening of a non-selective pore in the mitochondrial inner membrane, commonly known as the MPT pore<sup>45-47</sup>. The main components of the MPT pore are thought to be the adenine nucleotide translocase (ANT), voltage-dependent anion channel (VDAC) and cyclophilin D (CypD)<sup>16</sup>. MPT induction results in osmotic swelling of the mitochondrial matrix, uncoupling of mitochondria, rupture of the OMM and the release IMS proteins, including cytochrome *c* and AIF into the cytosol. Yet, the contribution of MPT pore in apoptosis is still debated as the elimination of the VDAC isoforms (one isoform, different combinations of two isoforms, or all three isoforms), either by genetic knockout or by knockdown using small interfering RNAs (siRNAs), did not influence MPT *in vitro* as well as caspase cleavage and cell death<sup>48</sup>.

In contrast, the deletion of both ANT1 and ANT2 genes resulted in increased resistance to  $\text{Ca}^{2+}$ -induced MPT *in vitro*. Conversely, cells deficient in both ANT1 and ANT2 remain responsive to several triggers of apoptosis. This finding questions the potential link between their involvement in MPT induction and cell death<sup>49</sup>. The only *bona fide* component/modulator of MPT appears to be the CypD, since CypD-deficient cells are more resistant to MPT induction and cell death in comparison to wild type (wt) cells<sup>50</sup>. Interestingly, CypD-deficient cells died normally in response to various apoptotic inducers, but remained resistant to necrosis<sup>50,51</sup>. Therefore, the role of MPT as a possible mechanism to induce MOMP in apoptotic cell death is still a matter of debate.

It has long been recognized that the Bcl-2 family of proteins play a prominent role in the regulation and execution of OMM permeabilization. In 1997, some early indications of the importance of the Bcl-2 proteins in regulating the release of cytochrome *c*, were obtained by two independent groups. They observed that the overexpression of Bcl-2 prevented mitochondrial release of cytochrome *c* as well as the initiation of apoptosis<sup>52,53</sup>. In addition, there was complete inhibition of cell death when Bcl-X<sub>L</sub> was co-expressed with Bcl-2<sup>54</sup>. Bax was also shown to directly stimulate cytochrome *c* release in isolated mitochondria<sup>55</sup>. In 2000, the double knockout of Bax and Bak was made in mice by Korsmeyer’s group. Cells derived from these mice were resistant to the vast majority of known apoptotic stimuli, including growth factor deprivation, staurosporine, etoposide, ultraviolet radiation and the ER stress-inducing stimuli, thapsigargin and tunicamycin<sup>21</sup>. Hence, the predominant role of Bax and Bak was established as the main mechanism of OMM permeabilization regulating the

mitochondrial release of pro-apoptotic proteins was established. Much work has been dedicated to these mediators of OMM permeabilization, Bax and Bak, ever since. These proteins share structure similarities and consist mainly of  $\alpha$ -helices as well as three BH-domains. Importantly, Bax and Bak differ in their subcellular localization. During apoptosis, both proteins change conformation and insert two central helices into the membrane. In these conformations they can assemble into higher oligomers to promote formation of the pores through which the pro-apoptotic proteins of the IMS are released. Although these conformational re-arrangements are already quite complex for Bak, the situation with Bax is even more intricate, because apart from conformational changes it must also be selectively targeted to the OMM<sup>26</sup>. Therefore, the activation and OMM targeting of Bax have to be precisely controlled and regulated to ensure apoptosis execution. In order to translocate to the mitochondria, cytosolic Bax must be activated by specific mechanisms. As mentioned above, two different, but not mutually exclusive, concepts have been elaborated; direct or indirect activation. The direct activation of Bax is thought to be mediated by a BH3-only protein like the Bim, Puma or the truncated form of Bid (tBid). The interaction of their BH3-only domain with Bax is believed to direct Bax to the mitochondria followed by its insertion into the OMM, oligomerization and pore formation. The second model, indirect activation, derived from the fact that all BH3-only proteins need to bind at least some pro-survival members of the Bcl-2 family to have some pro-apoptotic activity. Mutations that disrupt the binding also impaired the pro-apoptotic activity of these proteins. The effector proteins Bax/Bak are kept in check by the pro-survival proteins Bcl-2/Bcl-X<sub>L</sub>, until BH3-only proteins functionally neutralize the pro-survival proteins allowing Bax/Bak to oligomerize and permeabilize the OMM. There is no interaction between the BH3-only proteins and Bax/Bak according to this hypothesis<sup>25</sup>.

However, the precise mechanism by which Bax targets the mitochondria, and what “receptor” it binds is still unclear. Some potential candidates have been described, among them VDAC<sup>56</sup>, Tom22, a receptor of the translocase of the outer membrane (TOM) complex<sup>57</sup>, the fission/fusion machinery,<sup>58</sup> or cardiolipin<sup>59</sup>.



**Figure 2. Molecular mechanisms of mitochondrial outer membrane permeabilization during cell death.**

### Apoptosis-Inducing Factor

The *AIF* gene is spread-out over 16 exons and resides on the human chromosome X (Xq25-Xq26). The most abundant *AIF* transcript (*AIF1*) is translated in the cytoplasm and imported into the mitochondria with the help of its N-terminal MLS as a 67 kDa precursor protein. The C-terminal part of the MLS, which is encoded by the second exon (residues 36 to 82), harbors a TM region (residues 66 to 84) that functions as an inner membrane sorting signal (IMSS) and is important for targeting of AIF towards the inner mitochondrial membrane. As the imported and fully processed protein is inserted into the inner membrane facing the intermembrane space as 62 kDa protein<sup>60</sup>, it adopts its mature folding through incorporation of its co-factor flavin adenine dinucleotide (FAD)<sup>61</sup>. In healthy cells, AIF exerts NADH oxidase activity and is located close to Complex I of the respiratory chain<sup>62</sup>. Accordingly, depletion of AIF compromises oxidative phosphorylation in a variety of *in vivo* experimental model systems<sup>63</sup>. The crystal structure of mouse and human AIF shares 92% identity and does indeed reveal an oxidoreductase-like folding of the protein<sup>64</sup>. A complete deficiency of AIF results in embryonic lethality, indicating its important physiological role<sup>65</sup>.

The Harlequin (Hq) mouse model exhibits a reduced AIF expression, 10–20% of normal level due to a retroviral insertion into the *AIF* gene. In contrast to AIF-deficient embryos, these mice are viable but develop blindness, ataxia and progressive loss of retinal and cerebellar neurons<sup>65</sup>. It was proposed that AIF might be involved in the

detoxification of ROS as the Hq mice are sensitive to oxidative stress<sup>66,67</sup>. Later, this idea was challenged as a reduction (by either knockout or knockdown) of the AIF expression level was found to lead to either an increase or a decrease in ROS production<sup>68,69</sup>. More recently, using Hq mouse brain mitochondria, it was shown that AIF does not directly regulate ROS production in the mitochondria<sup>70</sup>. Thus, the functional connection between AIF and ROS production remains elusive.

Upon treatment with certain apoptotic stimuli, AIF can be cleaved from its membrane anchor by proteases, including calpains and cathepsins, generating a soluble 57 kDa AIF fragment. This fragment can be released from the IMS into the cytosol upon permeabilization of the OMM. Since AIF harbors two nuclear localization signals (NLS) to translocate into the nucleus<sup>60</sup>. Here, it contributes to large-scale DNA fragmentation and chromatin condensation, although the precise mechanism(s) of the nuclear function of AIF are unknown<sup>71</sup>. The nuclear translocation requires its obligate partner cyclophilin A (CypA). Accordingly in CypA-depleted neurons exposed to hypoxia-ischemia, AIF remained in the cytosol after being liberated from the mitochondria. If AIF cannot enter the nucleus, it does not induce cell death<sup>72</sup>. Hence, the release of AIF from mitochondria *per se* is not sufficient to explain its function in the apoptosis.

Finally, while it is clear that the AIF-mediated pathway is not necessarily involved in all forms of apoptosis, but appears to be an important cell death mechanism only in certain cell types<sup>73</sup>. Hence, the elimination of AIF does not protect from apoptosis induced by most drugs, and in contrast to findings with caspases, overexpression of AIF does not trigger cell death. This indicates that AIF is not a universal death effector protein. Further, knockout of any one of the caspases results in an increase in cell number. In contrast, genetic inactivation of AIF in mice was embryonically lethal but, surprisingly, no excess in cell number was observed. All current *in vivo* observations suggest that AIF might be critical for the death of neurons, and cells of neuroendocrine origin, such as certain lung cancer cells, as well as for retinal degeneration.

### **Role of Ca<sup>2+</sup> in cell death signaling**

Ca<sup>2+</sup>, as a messenger, is essential for cell viability and is involved in multiple physiological processes, such as transcription, cell migration, contraction, secretion, metabolism and cell death<sup>74,75</sup>. In general, physiological Ca<sup>2+</sup> responses are of a transient nature as Ca<sup>2+</sup> oscillations. The intracellular Ca<sup>2+</sup> level is tightly controlled and the resting cytosolic concentration is 100-200 nM as compared to the high 1-2 mM concentration in the extracellular milieu. This creates a huge electrochemical gradient for Ca<sup>2+</sup> ions over the membrane<sup>76</sup>.

Cellular Ca<sup>2+</sup> overload in a cell can result from the import of Ca<sup>2+</sup> from the extracellular store or from excessive release from the intracellular stores, ER and mitochondria. ER is the main intracellular store and there are multiple Ca<sup>2+</sup> channels and pumps including regulating its Ca<sup>2+</sup> concentration. Among them are the sarco(endo)plasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA), inositol-1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) receptors (Ins(1,4,5)P<sub>3</sub>Rs) and ryanodine receptors (RYRs). In addition, Ca<sup>2+</sup>-binding proteins (calreticulin, calsequestrin) can sequester Ca<sup>2+</sup> in the ER<sup>77</sup>.

The Ca<sup>2+</sup> handling system of the mitochondria consists of four main players, including the mitochondrial uniporter which accounts for the uptake of the ion into the mitochondrial matrix. The release of Ca<sup>2+</sup> from the organelle occurs through three

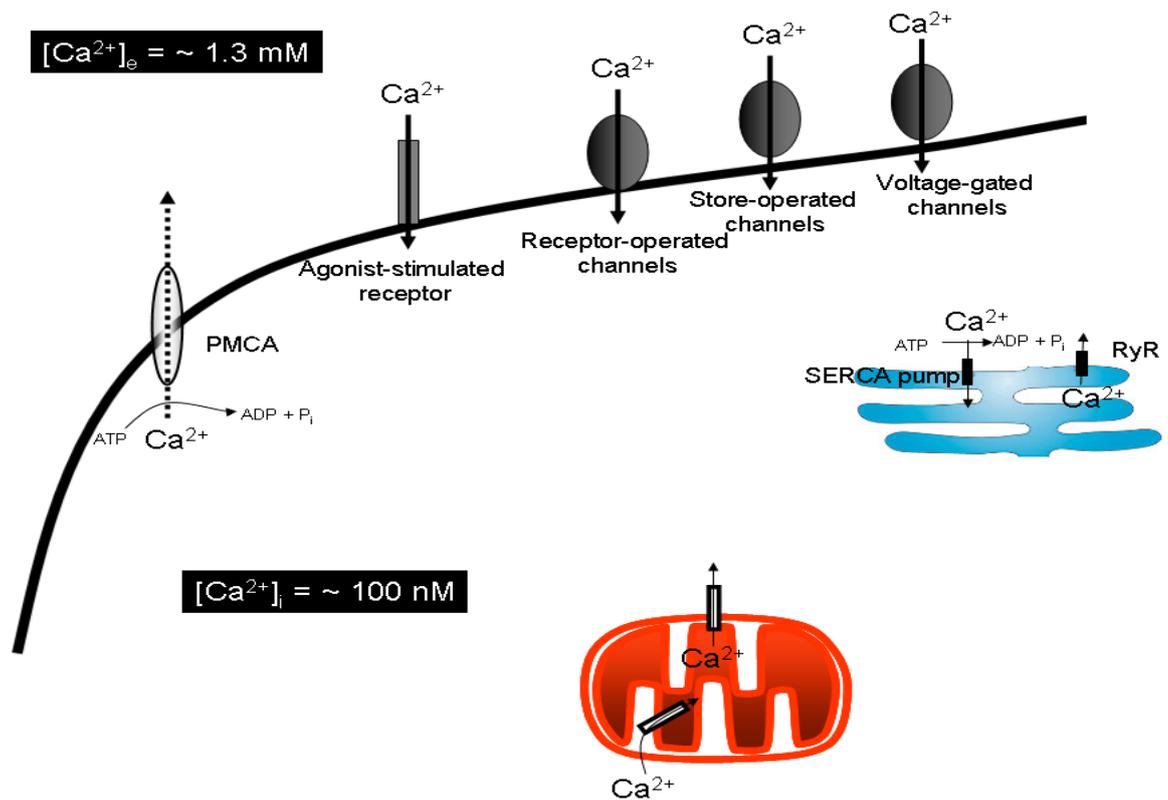
main pathways, namely, the permeability transition pore (PTP), reversal of the uniporter or via the  $\text{Na}^+/\text{H}^+$ -dependent  $\text{Ca}^{2+}$  exchange<sup>76</sup>.

Cellular  $\text{Ca}^{2+}$  import through the plasma membrane occurs largely by receptor-operated, voltage-sensitive and store-operated channels. The plasma-membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) and the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger regulate the  $\text{Ca}^{2+}$  extrusion from the cell and transports  $\text{Ca}^{2+}$  against its gradient in an energy dependent fashion<sup>78</sup>.

The role of  $\text{Ca}^{2+}$  as a death trigger was first suggested by A. Fleckenstein and colleagues, who proposed that cellular  $\text{Ca}^{2+}$  overload might be the mechanism underlying the cardiac pathology that occurs after ischemia<sup>79</sup>. Later, over-stimulation of receptors as well as some cytotoxic drugs were shown to result in detrimental  $\text{Ca}^{2+}$  overload. More recently, it has become clear that  $\text{Ca}^{2+}$  cannot only trigger cell death, but can also contribute to cell death at different stages of apoptosis and necrosis. Depending on the severity of the  $\text{Ca}^{2+}$  overload, it can lead to either apoptosis or necrosis<sup>78</sup>.

Today it is well known that  $\text{Ca}^{2+}$  signaling is interwoven in much of the apoptotic process. Some examples are listed below:

- a) Calpains are  $\text{Ca}^{2+}$ -dependent enzymes that have been reported to cleave multiple substrates during apoptosis including AIF, Atg5, neurofilaments, caspases, actin and PKCs among others as a part of the apoptotic pathway<sup>76</sup>.
- b) Mitochondrial  $\text{Ca}^{2+}$  overload can trigger MPT induction resulting in osmotic swelling of the mitochondria, and as a consequence, liberation of pro-apoptotic proteins. This might activate the caspase cascade or caspase-independent cell death.  $\text{Ca}^{2+}$  uptake into mitochondria has also been reported to stimulate mitochondrial ROS formation<sup>80,81</sup>.
- c) Activation of  $\text{Ca}^{2+}$ -dependent kinases/phosphatases, like calcineurin, might affect transcription and represents yet another a mechanism by which  $\text{Ca}^{2+}$  can regulate apoptosis<sup>75</sup>.
- d) Several endonucleases responsible for the degradation of DNA during the late step of apoptosis are activated by  $\text{Ca}^{2+}$ .
- e)  $\text{Ca}^{2+}$  is also involved in the final step of apoptosis when the phosphatidylserine is exposed on the cell surface as an “eat me signal” for macrophages<sup>76</sup>.



**Figure 3. The regulation of the intracellular  $\text{Ca}^{2+}$  homeostasis.**

### **Role of ROS in cell death signaling**

Mitochondria fulfill a crucial function in energy metabolism. Approximately 1-2% of the molecular oxygen consumed during physiological respiration is converted into superoxide radicals ( $\text{O}_2^{\cdot-}$ ), mainly at Complex I and Complex III of the respiratory chain. Superoxide serves as a precursor for most of the ROS formed. Superoxide dismutases transform superoxide into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).  $\text{H}_2\text{O}_2$  in turn can be transformed via the Fenton reaction into the highly reactive hydroxyl radical ( $\cdot\text{OH}$ ). Excessive ROS production can lead to oxidation of macromolecules, such as DNA, proteins, lipids and has been implicated in cell death, ageing, and mitochondrial and nuclear DNA mutations. Today mitochondria are considered as the main source of intracellular ROS production. Within the matrix of the mitochondria, there is an antioxidant defense system, including MnSOD, which converts superoxide into hydrogen peroxide. The latter can either diffuse into the cytosol, or be further metabolized by glutathione peroxidase and peroxiredoxin into  $\text{H}_2\text{O}$ . Glutathione, GSH, and several GSH-linked antioxidant enzymes together with the peroxiredoxins and the thioredoxin system represent the most prominent mitochondrial antioxidant protection systems<sup>82</sup>.

ROS can affect the induction of cell death by oxidizing macromolecules. Some examples of how this might occur are listed below:

## Lipids

Polyunsaturated fatty acid residues in phospholipids are extremely sensitive to oxidation. For example, specific peroxidation of cardiolipin has been reported to precede cytochrome *c* release from mitochondria during apoptosis<sup>83,84</sup>.

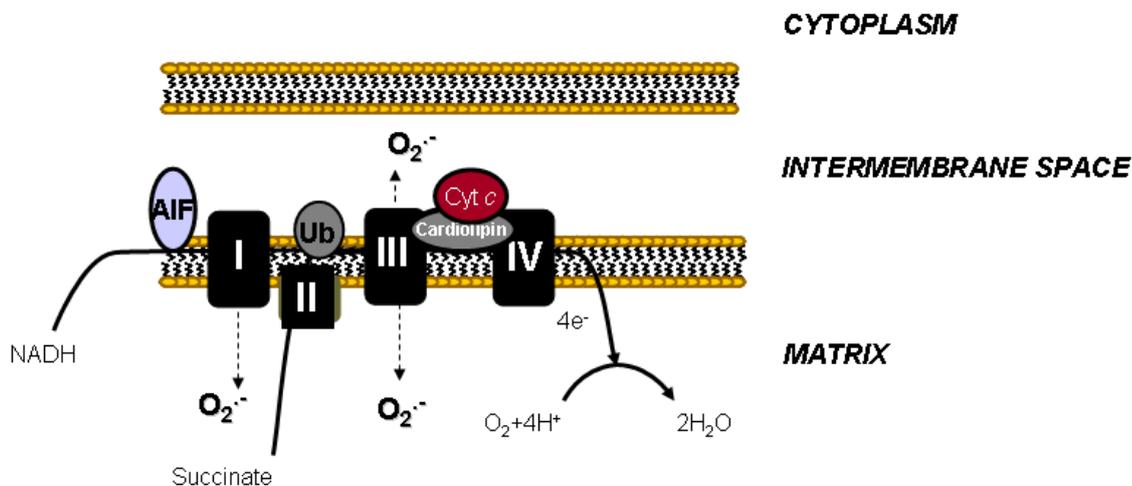
## Proteins

Protein oxidation is less characterized as compared to lipid oxidation, although several types of damage have been demonstrated, including oxidation of sulfhydryl groups, cross-linking of proteins, reactions with aldehydes and carbonylation. The latter is mediated by direct oxidation of certain amino acids including lysine, arginine, proline, and threonine. Carbonylation *per se* can dramatically alter the tertiary structure of a protein, leading to its partial or complete unfolding. Hence, oxidation of proteins can cause loss of their normal functions, e.g., enzymatic activity, channel forming properties, etc., and to enhanced degradation<sup>82</sup>.

Oxidation of ANT, a component of the MPT pore, has been shown to have cysteine groups that are sensitive to oxidation, which facilitates MPT induction<sup>85</sup>.

## DNA

Oxidative damage to DNA causes modification of the backbone of DNA. Single and double strand-breaks, as well as cross-links to other molecules have been reported. Many of these DNA modifications are mutagenic and contribute to ageing and cancer<sup>82</sup>.



**Figure 4. Formation of mitochondrial reactive oxygen species (ROS) by the respiratory chain.**

## The present study

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### Goal of the research

This project focuses on the investigation of the role of mitochondria in regulation of apoptotic cell death. Dysregulation of apoptosis plays an important role in the pathogenesis of many human diseases, including cancer, autoimmune and neurodegenerative disorders.

Specifically, the release of multiple pro-apoptotic proteins, that are normally present in the IMS of these organelles, has been shown to be a crucial event during the early stages of the apoptotic process. The biochemical machinery involved in the apoptotic killing and dismantling of the cell is accessible to activation by a variety of signals. Although it appears that distinct cell death pathways can be triggered by different signals, they often merge at a common regulator of this multistep process. Currently, it is widely accepted that the mitochondria serves as such a regulator. Therefore, the main goal of this project was to investigate the involvement of mitochondria in cell death.

The specific aims:

1. To determine the target specificity of pro-apoptotic Bax in the outer mitochondrial membrane.
2. Investigation of mechanisms of protein release from the mitochondrial intermembrane space.
3. Investigation of molecular mechanisms regulating in the cleavage and release of Apoptosis-Inducing Factor from the mitochondria.

## Materials and methods

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Detailed descriptions of the techniques used, can be found in papers I-IV presented in this thesis. Below is a brief list of the methods used:

### **Culture of bacteria, yeast, mammalian cell lines**

All cell lines used were grown either in DMEM or RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin and 2% (w/v) glutamine and grown in a humidified 5% CO<sub>2</sub> atmosphere at 37°C allowing exponential growth. Bacterial cultures were grown in LB medium at 37°C. Yeast cultures were grown in a lactate medium at 30°C, except for the strains *tom40-4* and *tom40-2* and the wild type, which were grown at 25 °C.

### **Drugs and chemicals**

STS was discovered approximately 30 years ago, and it was found to be an inhibitor of protein kinase C (PKC). Later, it was shown to be a rather unspecific PKC-inhibitor with many targets. Therefore, several analogues of STS were manufactured, including N-benzoyl staurosporine (PKC412) and 7-hydroxystaurosporine (UCN-01). These two analogues are in clinical trials for different cancers types, including lung cancer.

### **Isolation of mitochondria**

Male Harlan Sprague-Dawley rats (6–8 weeks old) were killed by CO<sub>2</sub> inhalation in accordance with the European directive systems of protection of vertebrate animals for scientific research. The liver of male Sprague-Dawley rats were minced on ice, resuspended in MSH buffer (210 mM Mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.5) with 1 mM EDTA and homogenized with a dounce homogenizer and Teflon pestle. Homogenates were centrifuged for 8 min at +4°C 600 x g. Supernatant was decanted and re-centrifuged for 15 min at 5500 x g. The mitochondrial pellet was resuspended in MSH buffer at a protein concentration of 80–100 mg/ml. Rat liver mitochondria with a respiratory control ratio (defined as the rate of respiration in the presence of adenosine diphosphate (ADP) divided by the rate obtained following the expenditure of ADP) above 4 were used in all experiments. Fresh mitochondria were prepared for each experiment and used within 4 h.

### ***In vitro* transcription translation of proteins**

The TNT® Coupled Reticulocyte Lysate System (Promega) was used for the preparation of [<sup>35</sup>S]-radiolabeled proteins. Reticulocytes, being the progenitor cells of erythrocytes show high level of gene expression. The lysate was supplemented with an amino acid mixture without methionine, [<sup>35</sup>S]methionine, RNA polymerase and RNAsin (ribonuclease inhibitor) in the *in vitro* reaction. The mixture was incubated for 90 min at 30 °C. The efficiency and specificity of the reaction was analyzed by Western Blot and autoradiography.

### **Import of precursor proteins into mitochondria**

Precursor proteins were synthesized in reticulocyte lysate (TnT, Promega) and labeled with [<sup>35</sup>S] methionine. Import experiments were performed in import buffer (600 nM Sorbitol, 50 mM Hepes-KOH, 80 mM KCl, 10 mM MgCl<sub>2</sub>, 2.5 mM EDTA, 2 mM K-P<sub>i</sub>, 1 mM MnCl<sub>2</sub>, 1 mM ATP, 5 mM succinate, 5 mM NADH and [<sup>35</sup>S]-labeled import substrate pSuDHFR. Samples were incubated at room temperature for 20 min. The samples were subsequently divided into two fractions whereof one was treated with 5 mg/ml trypsin for 30 min on ice (degradation of non-imported proteins). Proteolysis was stopped by the addition of 5 mg/ml trypsin inhibitor (STI). Samples were fractionated and the pellet was resuspended in SH buffer and Laemmli's buffer was added prior SDS-PAGE analysis.

### **Expression and purification of recombinant proteins**

Full-length human Bax and human tBid were expressed in *Escherichia coli* strain (BL21 DE3) as N-terminal hexahistidine-tagged proteins from the vectors pET23Bax and pET23tBid, respectively. The proteins were purified in the absence of any detergent, using standard Ni<sup>2+</sup> chromatographic techniques. The proteins were dialyzed against 30% glycerol, 100 mM NaCl, 0.2 mM EDTA, 25 mM HEPES/KOH, pH 7.4, and stored at -80°C. To obtain oligomeric Bax, the recombinant, monomeric Bax was incubated prior to the experiments with 1% octylglycoside for 1 h at 4°C

### **Gel electrophoresis and immunoblotting**

Proteins from each sample were mixed with Laemmli's loading buffer, boiled for 5 min, and subjected to 12% SDS-PAGE at 40 mA followed by transfer to nitrocellulose membranes for 90 min at 120 V. Membranes were blocked for 30 min with 5% non-fat milk in Tris-buffered saline (TBS) at room temperature and subsequently probed with the desired primary antibody. The primary antibodies were diluted in TBS containing 1% bovine serum albumin, 0.05% Tween 20, and 0.1% NaN<sub>3</sub>. Secondary antibodies were diluted in 2.5% blocking buffer. Horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce Chemical Co. Blots were visualized by ECL (Amersham Biosciences).

### **Immunocytochemistry**

Cells were seeded on coverslips, fixed for 20 min in 4% formaldehyde at 4°C and washed with PBS. Incubations with primary antibodies, diluted (1:400) in PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (BSA), and secondary antibodies (1:200) were performed at 4°C overnight in a humid chamber and at room temperature for 60min, respectively. Nuclei were counterstained with Hoechst 33342 (10µg/ml in PBS solution) by 5 min incubation at room temperature. Between all steps, cells were washed for 3 × 10 min with PBS. Secondary FITC-conjugated antibodies directed to mouse (Alexa 488) or rabbit (Alexa 594) were purchased from Molecular Probes. Stained slides were mounted using Vectashield H-1000 (Vector Laboratories Inc.) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Zeiss).

### **RT-PCR**

Total RNA were isolated from U1810 or H661 cells using the RNeasy kit (Qiagen). The quality of the RNA was controlled by gel electrophoresis of 18S and 28S

ribosomal RNA. For the first strand cDNA synthesis the RevertAid™ M-MuL V RT enzyme (Fermentas) was used in combination with gene-specific primers according to the manufacturer's instruction. The PCR was performed using the Platinum® Pfx DNA polymerase system (Invitrogen) and the desired primers (Invitrogen): The conditions for HCN2 and G3PDH PCR reactions were: 94°C for 1 min (hot start) followed by 95°C for 1 min, 55°C for 30 s and 72°C for 1 min. For the PCR of HCN4, 57°C was used as annealing temperature. Samples were removed every fifth cycle to ensure exponential growth of the PCR product which was then analyzed on a 1% agarose gel.

### **Site-directed mutagenesis**

The pcDNA3.1-mHCN2 plasmid was kindly provided by Prof. Hang-Gang Yu (West Virginia University School of Medicine). Quick-change II XL Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's instructions to generate the point mutations of putative protein kinase C phosphorylation sites within the regulatory C-terminal domain of HCN2: T531A, T549A, T566A, and S818A. Desired substitutions were confirmed by sequencing.

### **Detection of protein carbonyls**

Protein carbonylation were detected using the OxyBlot™ protein oxidation detection kit (Chemicon International) according to the manufacturer's instructions. Briefly, AIF was immunoprecipitated and carbonyls were derivatized with 2,4-dinitrophenylhydrazine (DNPH). The mixture was subjected to 12% SDS-PAGE and Western blotting. Membranes were stained with Ponceau red to confirm equal loading. Immunoblotting was performed using anti-DNP antibody.

### **Transfection methods**

Plasmids were transfected using Lipofectamine2000 (Invitrogen) according to manufacturer's instruction. siRNA were transfected using INTERFERIN siRNA transfection reagent (Polyplus transfection). The level of the mRNAs was monitored by RT-PCR and the level of protein by Western Blot.

### **Flow cytometry**

FACS was used for several distinct analyses as follows:

- a) Analysis of expression/activation of proteins (Desired antibodies).
- b) Investigation of the polarization of plasma membrane (DiBAC).
- c) Ca<sup>2+</sup> measurements (Fluo-4/AM).
- d) Cell death assessment (AnnexinV/PI).

### **Ca<sup>2+</sup> measurements**

To perform Ca<sup>2+</sup> or polarization measurements, the cells were incubated (30 min at 37°C in 5% CO<sub>2</sub>) in KREBS medium containing 5 μM Fluo-4/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes). The HEPES medium contained 130 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES (pH 7.4) and 5 mM dextrose. During the time-lapse experiments all drugs were bath-applied and examined under a Zeiss LSM 510 META scanning laser confocal microscope (Zeiss).

Cells were loaded with Fluo-4/AM as described above and treated with desired drugs prior FL-1 FACS analysis of 10,000 cells.

### **ROS measurements**

Analysis of H<sub>2</sub>O<sub>2</sub> production by mitochondria was performed using a genetically encoded mitochondrial-targeted fluorescent indicator pHyPer-dMito (Evrogen). The fluorescent protein was inserted into the regulatory domain of *E. Coli* OxyR transcription factor. Binding of H<sub>2</sub>O<sub>2</sub> to the OxyR protein leads to the formation of disulfide bonds causing changes in fluorescence. Cells were seeded on coverslips and on the following day, transiently transfected with pHyPer-dMito using the Lipofectamine2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. All drugs were bath-applied and were performed using a Zeiss LSM 510 META confocal laser scanning microscope (Zeiss).

### **Apoptosis detection**

#### a) Nuclear condensation

To investigate cells undergoing apoptosis, nuclei were stained with DAPI or Hoechst and condensed/fragmented nuclei were counted. Briefly, cells were exposed to a drug, stained with Hoechst and fixed using 4% PFA.

#### b) Caspase processing

Detection of the pro- and processed form of caspases was performed using immunoblotting.

#### c) Caspase activity

The measurement of cleavage of caspase substrates (Peptide Institute, Osaka, Japan) was performed as follows:  $2 \times 10^6$  cells were washed once with PBS, resuspended in 25  $\mu$ l of PBS and placed on a microtiter plate. Cells were subsequently mixed with DEVD-AMC (50  $\mu$ M), dissolved in standard reaction buffer (100 mM HEPES, pH 7.25, 10% sucrose, 10 mM DTT, 0.1% CHAPS). Cleavage of the fluorogenic peptide substrate was monitored by AMC liberation in a Fluoroscan II plate reader (Thermo Electron Co., Waltham, MA, USA) using 355 nm excitation and 460 nm emission wavelengths.

#### d) Mitochondrial pro-apoptotic protein release

Cells were treated, harvested, washed in PBS and resuspended in SHKCL buffer (600 mM sorbitol, 125 mM KCl, 25 mM HEPES/KOH (pH 7.4)). Cells were permeabilized by the addition of 0.005% digitonin and incubated for 5 min at room temperature. Subsequently, cells were fractionated by centrifugation 13000 rpm for 5 min. The mitochondrial fraction (pellet) and cytosolic fraction (supernatant) were analyzed using immunoblotting.

#### e) FACS analysis of AnnexinV/PI stained cells

Cell death was monitored using AnnexinV-FLUOS staining kit (Roche) according to manufacturer's instruction.

## Summary of the papers

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### Paper I

Ott M, **Norberg E**, Walter KM, Schreiner P, Kemper C, Rapaport D, Zhivotovsky B, Orrenius S. The mitochondrial TOM complex is required for tBid/Bax-induced cytochrome *c* release. *J Biol Chem.* 2007; 282,27633-27639.

Mitochondrial cytochrome *c* release is considered as a key event during apoptotic signaling that is regulated by Bcl-2 family proteins. The truncated form of Bid, tBid, is able to promote the oligomerization/insertion of Bax into the mitochondrial outer membrane leading to MOMP. Bax, a monomeric protein in the cytosol, is targeted by a yet unknown mechanism to the mitochondria. Several hypotheses have been elaborated to explain this targeting specificity and among the proposed targets are the mitochondrial porin (voltage-dependent anion channel), cardiolipin and more recently Tom22, a receptor of the translocase of the outer mitochondrial membrane (TOM) complex.

In paper I, we have systematically investigated components of the mitochondrial outer membrane that might be required for tBid/Bax-induced cytochrome *c* release by using mitochondria isolated from different mutants of the yeast *Saccharomyces cerevisiae* and recombinant Bax and tBid. We could show that exposed domains of the mitochondrial outer membrane proteins, like Tom20, Tom22, and Tom70, are not involved in tBid/Bax targeting on the OMM. Further, by isolating mitochondria from yeast lacking the gene for cardiolipin synthase, *CRDI*, or the mitochondrial porins, we could show that this deficiency did not affect the capability of tBid/Bax to permeabilize the OMM.

Next, we analyzed if other mitochondrial  $\beta$  barrel proteins could possibly contribute to the targeting of tBid/Bax to mitochondria. Hence, the role of Tom40, the pore-forming subunit of the TOM complex, was investigated using yeast mutants expressing a temperature-sensitive version of Tom40.

By employing several distinct approaches we could show that Bax insertion and the subsequent tBid/Bax-induced cytochrome *c* release were significantly reduced when the functionality of the Tom40 protein was perturbed. Further, we could show a direct interaction between Bax and the TOM complex. Antibodies directed against the TOM complex could interfere with the mitochondrial import machinery as well as Bax insertion and tBid/Bax-induced cytochrome *c* release in mammalian mitochondria. Thus, it appears that a functional TOM complex is required for the process of tBid/Bax-induced release of mitochondrial proteins.

## Paper II

**Norberg E, Gogvadze V, Ott M, Horn M, Uhlén P, Orrenius S, Zhivotovsky B.** An increase in intracellular  $\text{Ca}^{2+}$  is required for the activation of mitochondrial calpain to release AIF during cell death. *Cell Death Differ.* 2008; 15,1857-1864.

AIF can be liberated from the mitochondria during apoptosis and translocate to the nucleus, where it contributes to chromatin condensation and large-scale DNA fragmentation. The mechanism of AIF processing and release is not fully understood although it has been reported that such a cleavage can be catalyzed by  $\text{Ca}^{2+}$ -dependent calpain and by  $\text{Ca}^{2+}$ -independent cathepsins B, L, and S. Further, the subcellular localization of the enzyme responsible for this event remains elusive.

In paper II, we aimed to address these issues by using isolated mitochondria and NSCLC U1810 cells as model systems. We could observe processing of AIF upon addition of  $\text{Ca}^{2+}$  to highly purified mitochondria. In addition, we detected calpain-1 in the mitochondria and, accordingly, a protease-inhibitor screen revealed BAPTA/AM (a  $\text{Ca}^{2+}$  chelator) and a selective calpain inhibitor (PD150606) to be able to prevent the cleavage of AIF.

We subsequently investigated whether an apoptotic inducer (STS) could evoke a  $\text{Ca}^{2+}$  signal sufficient to promote calpain-mediated AIF processing in U1810 cells. The exposure of cells to STS provoked a prolonged increase in the intracellular  $\text{Ca}^{2+}$  concentration that lasted for several minutes. This sustained elevation of the intracellular  $\text{Ca}^{2+}$  level was required to trigger the proteolysis of AIF, while a transient  $\text{Ca}^{2+}$  response did not. Further, the  $\text{Ca}^{2+}$  signal evoked by STS originated from the extracellular space, since no  $\text{Ca}^{2+}$  response was observed when cells were exposed to STS in  $\text{Ca}^{2+}$ -free medium. To investigate whether calpains were activated upon STS treatment, the cleavage of atg5 was also analyzed. Indeed, a significant increase in the cleaved atg5 product could be detected. These data allowed us to conclude that a mitochondrial  $\text{Ca}^{2+}$ -activated calpain was the enzyme mediating AIF release from mitochondria during apoptosis.

### Paper III

**Norberg E, Gogvadze V, Vakifahmetoglu H, Orrenius S, Zhivotovsky B.** Oxidative modification sensitizes mitochondrial apoptosis-inducing factor to calpain-mediated processing. *Free Radic Biol Med.* 2010; 48,791-797.

Although processing of mitochondrial apoptosis-inducing factor (AIF) is essential for its function during apoptosis in most cell types, the detailed mechanisms of AIF cleavage remain elusive. Our recent findings indicate that the proteolytic process is  $\text{Ca}^{2+}$ -dependent and is mediated by a calpain located in the mitochondria. In addition, several recent publications reported that antioxidants can suppress AIF-mediated cell death in neurons and in cancer cells of various origins. Therefore, the aim of the study in paper III was to investigate the potential functional connection between AIF processing/release and reactive oxygen species (ROS). We could demonstrate that ROS formation stimulated at either Complex I or Complex III of the mitochondrial respiratory chain could significantly enhance the cleavage of AIF. We subsequently analyzed if stimulated ROS formation also could further enhance the cell death effect induced by STS. Pre-treatment with Antimycin A significantly stimulated STS-induced AIF-mediated death of U1810 cells.

By using live cell imaging we monitored the mitochondrial liberation of AIF-GFP, the  $\text{Ca}^{2+}$  responses and ROS accumulation and could show that the  $\text{Ca}^{2+}$  and ROS signals were linked. In fact, the ROS formation upon stimulation with STS, was  $\text{Ca}^{2+}$ -induced. Accordingly, pre-treatment with BAPTA/AM prevented ROS accumulation, while antioxidants did not affect the STS-induced  $\text{Ca}^{2+}$  response. In addition, we could observe that ROS formation increased the kinetics of AIF release and translocation to the nucleus.

Proteins are major targets for ROS, which can trigger several modifications of the protein structure. For instance, posttranslational modification by carbonylation might result in either partial unfolding, inactivation, or proteasomal degradation. Therefore, we investigated whether processing of AIF might involve its oxidative modification. While only a slight increase in overall protein carbonylation was observed, a selective carbonylation of AIF was detected, in STS-treated cells. We could further show that carbonylation of AIF preceded its cleavage and release. Finally, we demonstrated that the carbonylation made AIF more susceptible (5-fold) for calpain-mediated proteolysis. Combined, our data provide evidence that ROS-mediated, posttranslational modification of AIF is critical for its cleavage by calpain and consequently for AIF-mediated cell death.

#### Paper IV

**Norberg E**, Karlsson M, Korenovska O, Szydłowski S, Silberberg G, Uhlén P, Orrenius S, Zhivotovsky B. Critical role for hyperpolarization-activated cyclic nucleotide-gated channel 2 in the AIF-mediated apoptosis. *EMBO J.* 2010; 29,3869-3878.

Cellular calcium uptake is a controlled physiological process mediated by multiple ion channels. We previously showed that the exposure of cells to either one of the PKC inhibitors, staurosporine or PKC412, can trigger  $\text{Ca}^{2+}$  influx leading to cell death. However, the precise molecular mechanisms regulating these events remained obscure.

In paper IV, we examined which plasma membrane  $\text{Ca}^{2+}$  channels might be involved in the STS/PKC412 induced  $\text{Ca}^{2+}$  response. We found that STS/PKC412 evoked a hyperpolarization of the plasma membrane which was required for the prolonged import of  $\text{Ca}^{2+}$ . By using a genetic approach (siRNA), we observed that import occurred via the Hyperpolarization-activated Cyclic Nucleotide-gated channel 2 (HCN2). This influx of  $\text{Ca}^{2+}$  was shown to be sufficient to trigger caspase-independent AIF-mediated apoptosis. Importantly, the PKC inhibitors did not cause  $\text{Ca}^{2+}$  entry into HEK293 cells, which do not express the HCN channels. However, heterologous expression of mHCN2 could rescue the kinase inhibitor-induced  $\text{Ca}^{2+}$  response as well as sensitize them to STS/PKC412-mediated apoptosis. To investigate whether the observed STS/PKC412-induced  $\text{Ca}^{2+}$  influx was regulated via a phosphorylation-dependent mechanism, the cells were exposed to PKC activators. Pre-treatment of cells with either one of the two PKC activators, prior to administration of STS or PKC412, prevented the prolonged  $\text{Ca}^{2+}$  import via the HCN2 channel. STS/PKC412 caused a rapid dephosphorylation of HCN2 and the PKC activators could prevent this and as well as the prolonged  $\text{Ca}^{2+}$  influx. *In silico* analysis of the HCN2 channel revealed four conserved putative PKC phosphorylation sites within its internal C-terminus regulatory domain. Site-directed mutagenesis of these putative PKC phosphorylation sites revealed that dephosphorylation of Thr<sup>549</sup> was critical for the prolonged  $\text{Ca}^{2+}$  entry required for AIF-mediated apoptosis. Strikingly, this mutant could be activated and mediate prolonged  $\text{Ca}^{2+}$  influx upon hyperpolarization also in the absence of drugs, as no dephosphorylation events were required. We could further confirm this mechanism in primary culture of cortical neurons. Our findings demonstrate a novel role for the HCN2 channel, beyond its physiological function, by providing evidence that it can act as an upstream regulator of cell death.

## General discussion

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### Mechanisms of Bax activation

In order for Bax to translocate to the mitochondria during apoptotic signaling, the cytosolic Bax has to be activated by specific mechanisms. Two different concepts have been elaborated, which propose either direct activation of Bax by a BH3-only proteins (direct activation model) or the relief of inhibition of constitutively active Bax by sequestration of anti-apoptotic Bcl-2 family members (indirect activation model).

The prevailing concept of OMM permeabilization involves the interaction of Bax with a BH3-only activator protein, like tBid, Bim or Puma. The interaction of their BH3-only domain with Bax is thought to initiate the translocation of Bax to the mitochondria followed by its insertion into the OMM, its oligomerization and the release of IMS proteins. How such a direct activation would proceed at the molecular level remains elusive, although it is conceivable that the transient interaction of a BH3-domain with Bax could result in conformational changes of the Bax protein<sup>24</sup>. Interestingly, the BH3-domain of Bax is normally hidden in a hydrophobic groove that is occupied by the C-terminal  $\alpha$ -helix. This C-terminal  $\alpha$ -helix has been shown to be required for the targeting of Bax to mitochondria and represents a C-tail membrane anchor found in many other mitochondrial outer membrane proteins<sup>86</sup>. Once inserted into the membrane, the contact with the bilayer can induce conformational changes in the Bax structure, leading to the insertion of  $\alpha$ -helices 5 and 6 into the lipid bilayer. This re-arrangement in the protein structure allows homo-oligomerization to form the pores<sup>25</sup>. The evidence for this model originates mainly from studies using reconstituted systems, consisting of recombinant proteins and isolated mitochondria or artificial liposomes. One recent hypothesis proposed that membrane-bound tBid was required to activate Bax<sup>87</sup>. These findings suggest that tBid initiates the activation of Bax in close proximity to the membrane, starting the slower process of membrane insertion and oligomerization of Bax.

According to the indirect activation model, the binding of the anti-apoptotic Bcl-2 proteins by the BH3-only proteins is sufficient to trigger the activation and recruitment of Bax to the mitochondria. This assumes that Bax would be constitutively active in a healthy cell. This concept originated from the finding that the anti-apoptotic Bcl-2 proteins have higher affinity for BH3-only proteins than their pro-apoptotic relatives, Bax and Bak. Accordingly, the knockout of the BH3-only proteins Bid, Bim and Puma does not affect the activation of Bax and translocation during apoptosis<sup>30,88</sup>.

How could this controversy between the two divergent concepts of Bax activation be resolved? The main difference between them is that the theories are derived from experiments using diverse model systems. Work with the reconstituted systems consisting of liposomes and recombinant proteins, convincingly shows that an activating function of certain BH3-only proteins is required for Bax action. In contrast, the indirect model implies that Bax gets activated when its inhibition by anti-apoptotic proteins is relieved. This discrepancy strongly suggests that an important component required for Bax activation is lacking in the experiments using reconstituted systems<sup>89</sup>. By employing heterologous systems, this issue might be resolved. For instance, when Bax is overexpressed in *Drosophila*, or various yeast strains, Bax is toxic to the cells, also in the absence of activating BH3-only protein<sup>90,91</sup>. Accordingly, monomeric Bax

can trigger cytochrome *c* release from isolated yeast mitochondria<sup>92</sup>. This might be due to the absence of anti-apoptotic Bcl-2 proteins in the yeast mitochondria. Importantly, the release of cytochrome *c* from the isolated yeast mitochondria can be accelerated by the inclusion of tBid, indicating a stimulating effect of tBid on Bax<sup>92</sup>. Hence, the results with the heterologous systems suggest that, indeed, both models might be correct. Consequently, Bax can target and permeabilize mitochondria also in the absence of activating BH3-only protein(s). However, BH3-only proteins can potently accelerate the process of permeabilization. Mechanistically, it is conceivable that the C-terminal membrane anchor flips out of the hydrophobic pocket of Bax with low frequency during thermal breathing of the molecule, allowing Bax to target the mitochondria. An activating BH3-only protein might transiently bind to the BH3-domain of Bax and enhance its targeting efficiency by stabilizing a conformation of Bax with an exposed C-terminal membrane anchor. If correct, C-tail exposure of Bax (in the absence of tBid), and its activation, should be a temperature sensitive process. Indeed, at 37°C a substantial fraction of Bax is constitutively active in the absence of anti-apoptotic Bcl-2 proteins in the heterologous, but not in the reconstituted, system<sup>92</sup>. Therefore, we speculate that the difference between the heterologous and the reconstituted systems could be explained by the presence of a factor in the yeast mitochondria offering a high-affinity binding site that is missing in the reconstituted system. We anticipate that the missing factor is a component of the OMM.

### **What is mediating the selective targeting of Bax to the mitochondria?**

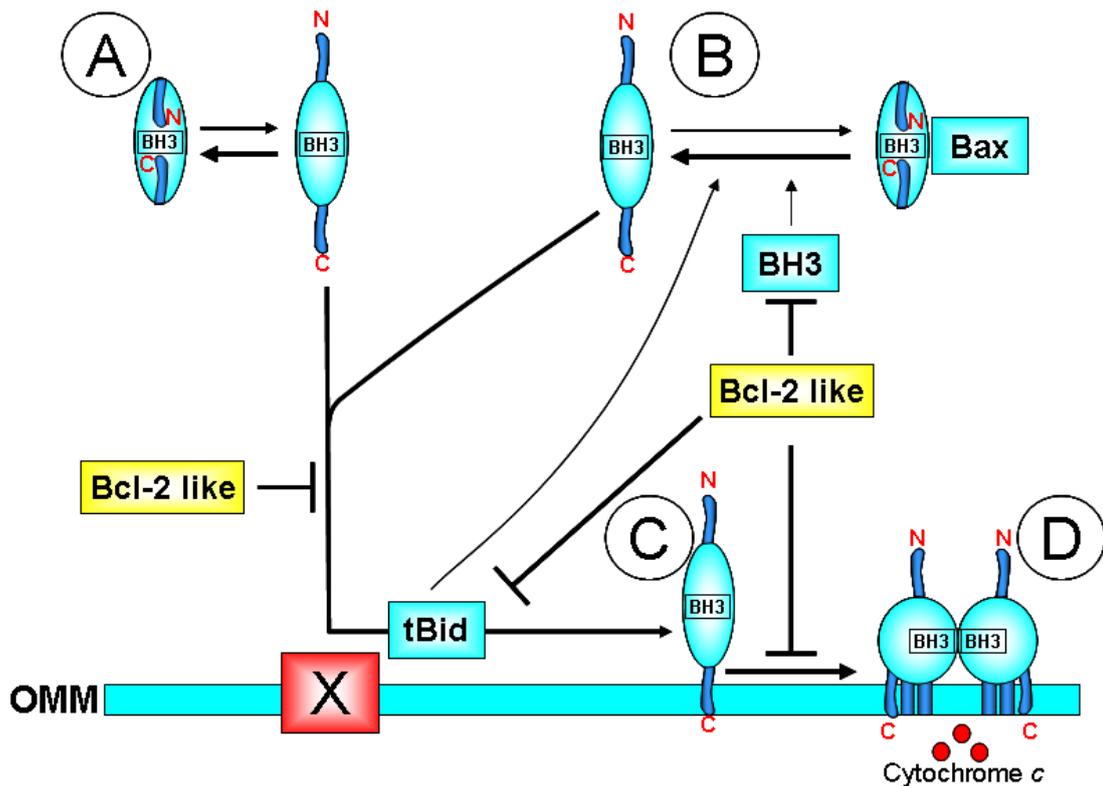
How does Bax selectively recognize the mitochondrial membrane in which to be inserted? The mitochondrial outer membrane houses, apart from its lipids, only a small number of proteins that can be divided into two classes: (1) membrane proteins with one or a few  $\alpha$ -helical transmembrane segments and (2)  $\beta$ -barrel membrane proteins. Bax is a member of the first group, displaying a similar pattern of positive charges at the side of the transmembrane segment that faces the intermembrane space, as found in other well-characterized single membrane spanning proteins.

The fact that the lipid composition of the OMM is very similar to that of the ER makes lipids in general as unlikely candidates. Otherwise, Bax should also permeabilize the ER and other biological membranes. The only phospholipid specific to the mitochondria is cardiolipin. A strict requirement of cardiolipin for targeting of Bax and tBid to the mitochondria has been suggested by experiments using reconstituted systems, consisting of artificial liposomes and recombinant proteins. However, most studies have concluded that there is very little cardiolipin in the OMM. Hence, cardiolipin makes up for only 0.3 and 1.4% of the total phospholipids in OMM, when determined for mitochondria derived from rat liver and yeast, respectively<sup>59,93</sup>. In contrast, in the studies with reconstituted liposomes, the concentrations of cardiolipin were ranging from 4–40% of the total phospholipids<sup>94</sup>. This exceeds by far the concentrations measured in the OMM fractions. Moreover, a strict requirement of cardiolipin for tBid/Bax-induced cytochrome *c* release has not been demonstrated in more physiological systems. The first of several suggested protein candidates for a mitochondrial Bax receptor was the voltage-dependent anion channel (VDAC1), also known as mitochondrial porin. Conversely, knockout of all VDAC isoforms 1, 2, and 3 in mice did not affect apoptosis signaling<sup>48</sup>. Another potential Bax receptor could be the mitochondrial fission and fusion machinery. It was found that GFP-Bax targets mitochondria at sites in which they divide e.g. on the mitochondrial foci that contain

Drp1, the dynamin mediating mitochondrial fission, and mitofusin 2 (Mfn2)<sup>58</sup>, another dynamin implicated in mitochondrial fusion. However, it has been shown that cells lacking both Mfn1 and Mfn2 undergo apoptosis in response to several stimuli, with similar, if not enhanced, kinetics as compared with control cells<sup>95,96</sup>. In contrast, a recent study reported that overexpression of Mfn1 protects cells from apoptosis; although one would expect that cell death would be enhanced if Mfn1 is a receptor for tBid/Bax<sup>97</sup>. Various components of the TOM complex have also been considered as possible Bax receptor candidates. Tom22, a central subunit of the translocase was found as interaction partner of the N-terminus of Bax in a yeast two hybrid-screen<sup>57</sup>. Subsequent analysis showed that injected antibodies against Tom22 can inhibit cell death efficiently in the glioblastoma multiforme cells. We have recently used a heterologous system consisting of yeast mitochondria and recombinant proteins to address the targeting specificity of Bax. Importantly, either the expression of Bax in yeast cells or the exposure of isolated yeast mitochondria to Bax and tBid, resulted in incorporation of Bax into the OMM and the release of cytochrome *c*. Hence, it appears that Bax can also insert into the OMM of yeast mitochondria<sup>92</sup>. As yeast mitochondrial proteins are different from their mammalian homologs, we anticipate that Bax insertion might occur through a conserved pathway for insertion of C-tail-anchored proteins<sup>89</sup>.

The family of  $\beta$ -barrel proteins is small, consisting of six different proteins in yeast and presumably also in mammals.  $\beta$ -barrel proteins are derived from bacterial outer membrane proteins and are found in non-plant eukaryotes exclusively in the mitochondrial outer membrane<sup>98</sup>. Altogether, this makes them conceivable candidates for proteins that would allow specific targeting of Bax to the mitochondria.

The TOM complex is a well-conserved 450 kDa protein assembly, equipped with receptors, structural components and a dimer of the Tom40 protein, resembling the general translocation pore through which precursor proteins are transported over the OMM. To date, all comparative analyses indicate that the general mechanism of protein import into mitochondria and, hence, probably, also that of the TOM complex *per se*, is conserved. Moreover, the function of the TOM complex might not be restricted to the translocation of precursor proteins across the OMM. It was recently suggested that this complex plays a direct role also in the outer membrane insertion of proteins with one transmembrane segment, like Bax with an exposed C-terminal<sup>99,100</sup>. The same group that suggested Tom22 as a potential receptor for Bax, later reported that Bax targeting required both Tom22 and Tom40<sup>101</sup>. In addition, the first *in vivo* data in *Drosophila melanogaster* revealed that the TOM subunits were required for cell death<sup>102</sup>.



**Figure 5. Hypothetical model of conformational change-mediated activation of Bax.**

Bax resides in the cytosol as a soluble monomeric protein. During thermal breathing the C-terminus (harboring the BH3-domain of Bax) is exposed (A). In the absence of anti-apoptotic Bcl-2 family proteins, this is sufficient for Bax integration into the OMM and cytochrome *c* release. A BH3-only protein may bind to the BH3-domain of Bax to stabilize its conformation in the cytosol. Alternatively, tBid might recruit Bax to the OMM and activate it (B). Here, Bax recognizes its receptor (X) and is inserted into the OMM (C). Inserted Bax oligomerizes to form pores through which intermembrane space proteins are released (D). Anti-apoptotic Bcl-2 proteins inhibit this activation by direct binding and neutralizing the BH3-only proteins and, therefore, Bax insertion.

### Enzymes regulating the cleavage and mitochondrial liberation of AIF

Several years ago it was shown that, upon permeabilization of OMM, AIF is not liberated from the mitochondria simultaneously with soluble proteins like cytochrome *c* and Smac/DIABLO<sup>103</sup>. This suggested that, apart from OMM permeabilization, some additional signals are required for its appearance in the cytosol. It was also reported that recombinant calpain-I could cleave and release AIF from Bid-permeabilized mitochondria<sup>104</sup>. In another important study, a detailed biochemical analysis revealed the presence of three forms of AIF (67, 62 and 57 kDa). Hence, AIF is synthesized as a 67 kDa pre-protein and upon import into the mitochondria it is processed to the mature membrane-integrated form of 62 kDa. Thus, it appeared that AIF must be liberated from its membrane anchor in order to be released from the mitochondria<sup>60</sup>. Ever since, attempts were performed aiming to identify the enzyme(s) responsible for the release of AIF in cultured cells and *in vivo*. Two main candidates

have emerged, namely, calpains and cathepsins. Interestingly, both enzymes can truncate recombinant AIF in the same position, Gly102/Leu103<sup>105</sup>.

Recently, calpain-I was shown to harbour a mitochondrial localization signal and found to be present in the mitochondrial IMS<sup>44</sup>. Accordingly, we were able to detect calpain-I in a highly purified mitochondrial fraction and could further show that Ca<sup>2+</sup> addition to the mitochondria was sufficient to stimulate AIF cleavage<sup>106</sup>. In fact, most studies investigating the possible role of calpains in the AIF-mediated pathway have concluded that calpain-I is the most important enzyme involved in the processing of AIF<sup>73,104,106-110</sup>. More specifically, siRNA-mediated downregulation of either one of the two calpains revealed that only depletion of calpain-I affected AIF truncation<sup>110</sup>. Moreover, current data on AIF processing and release obtained from *in vivo* mice studies revealed that calpain-I was critical for the release of AIF<sup>109</sup>.

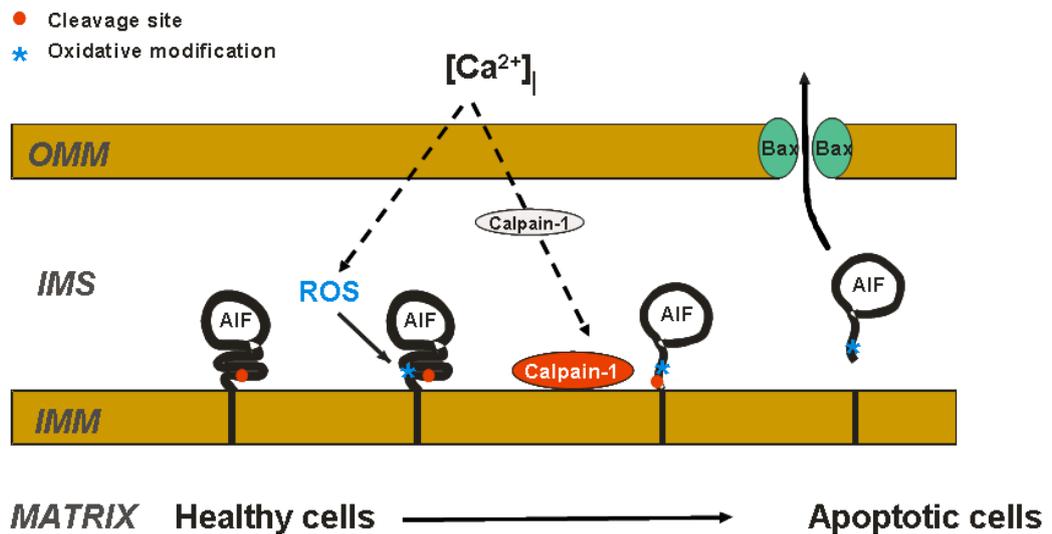
Ca<sup>2+</sup> is one of the most versatile cellular messengers that control physiological processes, such as the release of neurotransmitters and muscle contraction<sup>74</sup>. The physiological responses are normally governed by intracellular Ca<sup>2+</sup> transients. In contrast, toxic perturbation of the intracellular Ca<sup>2+</sup> homeostasis is often mediated either via an influx of extracellular Ca<sup>2+</sup> into the cell, or by a redistribution of Ca<sup>2+</sup> between different intracellular compartments, e.g. between the ER and the mitochondria. Toxic Ca<sup>2+</sup> elevation is also more pronounced and sustained when compared to physiological Ca<sup>2+</sup> signaling and lacks the oscillatory pattern typical of the latter<sup>76</sup>. For example, activation of calpain-mediated AIF release from the mitochondria during retinal degeneration was reported to require a sustained elevation of the intracellular Ca<sup>2+</sup> level<sup>111</sup>. Similarly, STS or PKC412 treatment of NSCLC cell lines led to an early import of extracellular Ca<sup>2+</sup> through the HCN2. This resulted in a sustained elevation of the cytosolic Ca<sup>2+</sup> level sufficient to cause calpain activation and subsequent AIF-mediated cell death<sup>106,112</sup>.

### **What else might modulate the processing of AIF?**

Determination of the crystal structure of both the murine and the human AIF proteins has revealed that the calpain/cathepsin cleavage site in AIF is not exposed on the surface of the protein, but rather embedded in its structure<sup>64,113</sup>. Therefore, one possible limiting step in AIF processing might be the accessibility of the cleavage site to the protease. Indeed, it was proposed that redox-regulated conformational changes of the AIF structure, affecting its binding to pyridine nucleotides, might influence the ability of calpain to cleave AIF in isolated mitochondria<sup>114</sup>. However, using the same approach we could not detect any protective effect of pyridine nucleotides on AIF processing. Hence, the binding of pyridine nucleotides to AIF does not seem to be a general phenomenon<sup>115</sup>.

Another possibility that might involve changes in the tertiary/quaternary conformation of AIF for its processing and release from the mitochondria is oxidative modification(s) triggered by ROS<sup>73</sup>. Indeed, there are several observations linking ROS and AIF, although the precise mechanism(s) of such interaction are still unclear<sup>66-69</sup>. For example, deletion of AIF can result in either an increase or a decrease in mitochondrial ROS production. It was proposed that AIF can function as a free radical scavenger and thereby prevent apoptosis<sup>66</sup>. In contrast, it was reported that mitochondria from both wild-type and Hq mice have the same capability to scavenge ROS<sup>70</sup>. Another study demonstrated that glutathione peroxidase-4 can sense and translate oxidative stress into AIF-mediated cell death<sup>116</sup>. Manganese superoxide

dismutase (MnSOD) was observed to protect from cell death in a mouse model of permanent focal cerebral ischemia. In this case, antioxidant-treated mice exhibited significantly less mitochondria/nuclear AIF translocation and large-scale DNA fragmentation<sup>117</sup>. Further, an AIF-mediated mechanism was shown to be critical for myocardial cell death upon ischemia–reperfusion. In this experimental model,  $\text{Ca}^{2+}$  and ROS signals were assigned as key mediators in regulating the release of AIF from mitochondria<sup>118</sup>. More recently, several studies have demonstrated that antioxidants can suppress AIF-mediated cell death in neurons as well as in NSCLC and other cancer cell lines<sup>119,120</sup>. In this scenario, ROS were able to promote the AIF cleavage and release. In line with these observations, we found that oxidative modification of AIF markedly (5-fold) increased its susceptibility to calpain-I-mediated processing. In addition, ROS stimulated also the mitochondria-nuclear translocation<sup>115</sup>. This led us to propose that oxidative modification might expose a normally hidden calpain cleavage site in AIF. Similar findings have been made earlier with a variety of calpain substrates<sup>121-127</sup>.



**Figure 6. Model of AIF processing during apoptosis.** AIF processing is sequentially regulated by both  $\text{Ca}^{2+}$  and ROS. The import of  $\text{Ca}^{2+}$  from the extracellular store in staurosporine or PKC412-treated cells leads to an elevation of the intracellular  $\text{Ca}^{2+}$  level for several minutes. Subsequently, the mitochondria sequester part of the  $\text{Ca}^{2+}$  increase during the normalization phase. The  $\text{Ca}^{2+}$  elevation governs at least two effects; first, a mitochondrial calpain located in the intermembrane space is activated by  $\text{Ca}^{2+}$  and second,  $\text{Ca}^{2+}$  uptake into mitochondria stimulates ROS production. This leads to oxidative modification (carbonylation) of AIF. Carbonylation of AIF increases the accessibility of the calpain cleavage site to proteolysis. Therefore, this conformational change makes AIF more susceptible to calpain-mediated processing. The soluble 57 kDa AIF is then released through Bax/Bak pores in the OMM.

## The role of AIF in apoptosis

As mentioned earlier, available experimental evidence show that AIF plays a critical role in cell death only in certain cell types, such as neurons and some tumor cells<sup>72,106,107,109,112,115,128-130</sup>. Furthermore, the type of apoptotic inducers also determines whether AIF will be important for the cell death process. Preferential AIF triggers should either directly perturb intracellular  $\text{Ca}^{2+}$  homeostasis, or lead to an early lysosomal permeabilization, in order to activate calpains or cathepsins that can liberate AIF from the mitochondria. Specifically the perturbation of the  $\text{Ca}^{2+}$  homeostasis is a frequent event during cell death signaling triggered by ischemia–reperfusion injury or after treatment with cytotoxic drugs.

An important indication of the significance of AIF for cell death is the timing of its processing and release in relation to other pre-apoptotic events. Indeed, as mentioned above mitochondria contain several pro-apoptotic proteins that are released from the intermembrane space during apoptotic signaling, including Smac/DIABLO, cytochrome *c* and AIF. These three proteins are of different size and are also located differently within the IMS. Smac/DIABLO is a soluble (25 kDa) protein, cytochrome *c* (14 kDa) is bound to cardiolipin, at the outer surface of the IMM by both electrostatic and hydrophobic interactions<sup>131</sup>, whereas AIF is anchored to the IMM with its transmembrane region<sup>60</sup>. Thus, it is reasonable to anticipate that the small molecular-weight and soluble proteins are released prior to AIF upon OMM permeabilization, since AIF requires an additional cleavage step before its liberation. However, if AIF cleavage would precede OMM permeabilization, solubilized AIF should be released early upon the loss of mitochondrial integrity. One example of this is seen in STS-treated lung carcinoma cells. In this model,  $\text{Ca}^{2+}$  is imported from the extracellular milieu and accumulates into the mitochondria, where it activates calpain-I to cleave AIF prior to its liberation from the IMM and release into the cytosol. Similar observations were made in several other studies using NSCLC cells treated with STS, or with a selective protein kinase C inhibitor, PKC412<sup>106,112</sup>.

As mentioned above, there are also many studies indicating the importance of AIF in mediating neuronal cell death. For instance, microinjection of neutralizing AIF antibodies, or siRNA downregulation of AIF, has been found to suppress glutamate-hypoxia- and NMDA-induced cell death in primary neurons<sup>107,109,132</sup>. Further, AIF knockdown in PC12 cells reduced the neurotoxic effects evoked by  $\text{MPP}^+$ <sup>133</sup>.

Many observations made *in vivo*, demonstrating the importance of the AIF-mediated pathway in neuronal cell death. For instance, as compared to wild-type mice, Hq mice were protected against NMDA- and kainic acid-induced neuronal damage in the hippocampus<sup>134</sup>. Cell death was also found to be suppressed in Hq mice subjected to hypoxia–ischemia<sup>128</sup>. Embryonic stem cells lacking AIF were less susceptible to serum withdrawal-induced apoptosis, as compared to wild-type ES cells<sup>71</sup>. Treatment of mice with a MnSOD mimetic (MnTBAP) prevented AIF translocation and DNA fragmentation upon ischemia and resulted in neuroprotective effects<sup>117</sup>. Neuroprotection was also observed in different mouse models, when AIF translocation was prevented by oral administration of HIV protease inhibitors<sup>135</sup>. Finally, it was shown that inhibition of the nuclear translocation of AIF was required to achieve neuroprotection in a rat model of retinal degeneration<sup>136</sup>.

## Conclusion

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In general, our results presented in paper I-IV provide information about different aspects of the mechanisms of release of proteins from the mitochondrial intermembrane space. In paper I, we could show that a functional translocase of the outer mitochondrial membrane (TOM) complex was required for Bax to target the mitochondria. The TOM complex represents a target that is uniquely expressed on the mitochondria and thus could conceivably mediate selective Bax insertion. While cardiolipin, mitochondrial porins, and cytosolic domains of outer membrane proteins appear not to be important for the targeting of Bax to mitochondria. The finding that Bax requires the TOM complex for insertion is reminiscent of a similar requirement for insertion of single membrane-spanning proteins, such as Tom20, Tom22, and Tom70, into the mitochondrial outer membrane.

In paper II, we found that staurosporine could evoke influx of  $\text{Ca}^{2+}$  from the extracellular space. This resulted in a prolonged  $\text{Ca}^{2+}$  elevation which was essential for AIF to become processed by a mitochondrial calpain during apoptotic signaling. We extended this study in paper III where we reported that the imported  $\text{Ca}^{2+}$  stimulated two separate events, i.e. the activation of mitochondrial calpain and the stimulation of mitochondrial ROS production. We could further show that the stimulated ROS production leads to oxidative modification (carbonylation) of AIF, which markedly increased its rate of cleavage by calpain. Combined, our data provide evidence that there is a sequential requirement for both  $\text{Ca}^{2+}$  and ROS accumulation required for liberation of AIF from the mitochondria. In paper IV, we characterized mechanisms underlying  $\text{Ca}^{2+}$  import into cells treated with the PKC inhibitors, PKC412 and staurosporine. We provide genetic evidence that the PKC inhibitors induced a prolonged  $\text{Ca}^{2+}$  import through the HCN2 channel in lung carcinoma cells and in primary culture of cortical neurons. Mutagenesis revealed that dephosphorylation of Thr<sup>549</sup> is critical for the prolonged  $\text{Ca}^{2+}$  influx and for AIF-mediated apoptosis. While the physiological functions of the HCN2 channels have been described, we demonstrate a novel role for this channel as an upstream regulator of cell death triggered by PKC inhibitors.

As for future studies, we will continue our investigation of the potential role of HCN2 channel as a novel drug target by analyzing its expression level in patient material. In parallel we will monitor tumor volume and tumorigenicity in athymic nude mice that have been transplanted with NSCLC cells and subsequently treated with PKC412 or vehicle controls. Moreover, parallel cohorts of mice will be transplanted with shRNA-HCN2-treated or mock-treated NSCLC cells and tumor growth will be monitored over time.

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