Chapter 14

Analysis of Pro-apoptotic Protein Trafficking to and from Mitochondria

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Abstract

Mitochondria play a key role in cell death and its regulation. The permeabilization of the outer mitochondrial membrane which is mainly controlled by proteins of the BCL-2 family, is a key event that can be directly induced by p53 and results in the release of pro-apoptotic factors to the cytosol, such as cytochrome c, second mitochondria derived activator of caspases/direct inhibitor-of-apoptosis (IAP) binding protein with low p*I* (SMAC/Diablo), Omi serine protease (Omi/HtrA2), apoptosis inducing factor (AIF), or endonuclease G (Endo-G). Hence, the determination of subcellular localization of these proteins is extremely important to predict cell fate and elucidate the specific mechanism of apoptosis. Here we describe the procedures that can be used to study the subcellular location of different pro-apoptotic proteins to be used in basic cell biology and toxicology studies.

Key words Mitochondria, Pro-apoptotic proteins, Cell fractions, Immunoblot, Immunoprecipitation, Immunocytochemistry

1 Introduction

Mitochondria are cellular organelles with important functions in cell life and death, acting as cellular powerhouses by producing energy to maintain cellular activity. However, mitochondria are also important checkpoints for cell fate decisions, playing a crucial role in programmed cell death (PCD) pathways. The permeabilization of the outer mitochondrial membrane (OMM) is a fundamental step in several tightly regulated pathways of cell death, allowing the release of proteins that are usually only present in the intermembrane space, and signaling cell death programs [1]. One of the best characterized types of cell death is apoptosis. Apoptotic signals may originate outside the cell (extrinsic pathway) or from any intracellular compartment (intrinsic pathway), constituting two distinct yet complementary apoptotic mechanisms. Both intrinsic and extrinsic stimuli may lead to OMM permeabilization.

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The OMM is selectively permeable to solutes, and its permeability and integrity are regulated by proteins of the BCL-2 family [2]. This family includes pro- and anti-apoptotic members, depending on the presence of different BCL-2 homology (BH) domains in their structure, conferring different functions [3]. Anti-apoptotic proteins, such as BCL-2 and BCL-xL have four different BH domains—BH1234. Pro-apoptotic proteins such as BAX and BAK are also multi-domain proteins with three different BH domains—BH123. BH3-only proteins are pro-apoptotic proteins with only one BH domain and can exert their pro-apoptotic function either by facilitating or by activating BH123 proteins, which then initiate OMM permeabilization. Facilitators or de-repressors, such as BAD, interact with BH1234 proteins, dissociating them from sequestered pro-apoptotic proteins, which become free to promote OMM permeabilization. The activators, such as tBID (which results from the cleavage of BID by caspase-8), directly activate BH123 proteins, either by stimulating the translocation of Bad to the OMM, or by interacting with BAK. OMM permeabilization may occur through a Bax/Bak-mediated mechanism [4] or by the opening of the mitochondrial permeability transition pore (MPTP) in the inner mitochondrial membrane [5]. In the latter mechanism, opening of the MPTP can lead to mechanical rupture of the OMM to which pro-apoptotic protein release follows or instead to the recruitment of pro-apoptotic proteins to the OMM by causing mitochondrial depolarization [6].

P53 is a redox-sensitive transcription factor with a broad range of actions, some of them related to survival and cell death [7]. In general, the tumor suppressor p53 exerts important roles in cell cycle progression and cell death coordinating multiple options for cellular response to genotoxic stress. p53 inhibits replication of the genome by blocking cell cycle progression at a G1/S check point in response to DNA damage [8]. In unstressed cells, p53 levels are low but its expression increase following stress signals acting through both transcription-dependent and -independent mechanisms to coordinate the appropriated cellular responses [9]. p53 activity depends on its ability to activate or repress gene transcription. Thus, p53 oscillates between latent and active sequence-specific DNA binding conformations and is differentially activated through posttranslational modifications including phosphorylation, acetylation and ubiquitination. On the other side, nonsequence-specific DNA binding may mediate other p53 actions [10]. In addition, p53 is also involved in mitochondrial-dependent cell death, collaborating in the execution of the apoptotic pathway. In this context, p53 undergoes a nuclear–cytoplasm–mitochondria trafficking and western blotting and immunocytochemistry are usually performed to detect the presence of p53 in nuclear, cytoplasmic, and mitochondrial extracts isolated as described in this paper. The analysis of immunoreactive bands in nuclear fractions

indicates whether nuclear translocation is occurring. The nuclear localization is critical for its transcriptional activity by activating genes that arrest cell growth and repair DNA damage. To further confirm its transactivation function, evaluation of the expression of the transcription target genes of p53 such as PUMA, NOXA, BAX, BID, and DRAM is often useful [11]. As described above, the activity of the p53 gene product is regulated by posttranslational modifications. These modifications of p53 affect its stability and can be a potential mechanism to select the target genes conferring differential binding affinity to the response elements. For example, acetylation influences p53 activity enhancing its transcriptional activity and can be detected using different acetyl-p53 antibodies available. Acetylation of p53 at carboxyl-terminal lysine residues enhances its transcriptional activity associated with cell cycle arrest and apoptosis. However, p53 acetylation at Lys-320/Lys-373/Lys-382 is also required for transcription-independent functions involving BAX activation [12]. The phosphorylation of p53 at Ser15 and Ser20 following DNA damage can also be detected by immunoblotting to evaluate its transactivation function since this phosphorylation promotes p53 activation and stabilization reducing the interaction between p53 and its negative regulator, the oncoprotein murine double minute 2 (MDM2) [13]. In addition, phosphorylation at Ser392 influences transcriptional activation of p53 and regulates the oncogenic function of p53 [14, 15]. MDM2 inhibits cytoplasmic retention of p53 by targeting it for ubiquitination and proteasomal degradation [16]. Some reports suggest not only that the cytoplasmic retention of p53 is able to repress autophagy [17, 18] but also that this retention can be mediated by acetylation since p53 acetylation reduces its ubiquitination status [19]. Although the precise molecular mechanisms behind this remain unclear, p53 deacetylases may be upregulated to mediate ubiquitination and degradation of p53 [20]. Because of this, the immunoblot analysis of cytoplasmic extracts can reveal whether p53 is accumulated in cytoplasm and can help to reveal the function of p53 in a specific context. p53 can promote cell death independently of transcription by two different mechanisms, each of which assigned to a specific localization of the protein: cytosol or mitochondrial. Both modes of action converge in the permeabilization of the OMM via activation of the pro-apoptotic proteins BAX or BAK. In fact, cytosolic p53 can directly activate BAX and thereby induce apoptosis [21]. On the other hand, in response to a broad spectrum of apoptotic stimuli, a fraction of p53 translocates to mitochondria and triggers a direct mitochondrial p53 death program [22]. For this, p53 physically interacts with the BCL-2 family member proteins BCL-xL and BCL-2 antagonizing their anti-apoptotic function and inducing OMM permeabilization [23]. Furthermore, mitochondrial p53 directly promotes the pro-apoptotic activities of BAX and directly induces

BAK oligomerization [24]. p53 also interacts with the antioxidant enzyme superoxide dismutase 2 (SOD2) leading to a reduction of its superoxide scavenging activity, and a subsequent decrease of mitochondrial membrane potential which contributes to the induction of pro-apoptotic mitochondrial alterations [25]. Surprisingly, p53 is released from mitochondria mediating a retrograde signaling pathway to the nucleus [26]. Therefore, the study of the subcellular localization of p53, its posttranslational modifications and the levels of p53-related/-targeted proteins are crucial to examine the p53 pathway and elucidate its particular role.

As described above, OMM permeabilization resulting from stress stimuli can result in simultaneous release of pro-apoptotic factors that are normally limited to the mitochondrial intermembrane or intercristae space, including cytochrome c, apoptosis-inducing factor (AIF), endonuclease G (endoG), Smac/Diablo, and Omi/HtrA2 [27]. Cytochrome c was first identified as being involved in mitochondrial bioenergetics, essential for the ATP production by oxidative phosphorylation, and later to apoptosis. Following the release of cytochrome c from the intermembrane space, it binds to Apoptotic protease activating factor 1 (APAF-1) and dATP [28], forming the apoptosome complex and initiating cell death with the activation of caspase-9 and consequent triggering of caspase cascade [29]. The second mitochondrial-derived activator of caspase (Smac), also known as direct IAP-binding protein with a low p*I* (Diablo) [29], resides in mitochondria in a mature form, and is released during apoptosis, which promotes caspase-dependent apoptosis by controlling the activity of inhibitor of apoptosis protein (IAP). Omi is a serine protease, which can be released from mitochondrial intermembrane space into cytoplasm, upon apoptotic insult. Omi cleaves both IAPs and cytoskeletal proteins, contributing to apoptosis in caspase-dependent and independent manners [30].

Independently of caspase activation, cell death can occur with the release of endoG and AIF. EndoG is also localized in intermembrane space bound to the inner membrane. Due to mitochondrial membrane potential loss, the mature EndoG is translocated to the nucleus and initiates oligonucleosomal DNA fragmentation. EndoG can regulate several mitochondrial enzymes expression, such as complex I (ND1 and ND2), complex IV (COX2), and complex V (ATPase6) [30] (Fig. 1). The AIF is a flavoprotein harboring NADH oxidase activity. Initially, the AIF was identified as a pro-apoptotic protein, inducing a type of programmed cell death independently of caspases activation. When AIF is added to purified nuclei extracts in a cell free system, chromatin condensation and large-scale DNA fragmentation to ~50 kbp fragments occurs [31]. Those effects were observed in intact cells after diverse apoptotic stimuli and also in models of retinal degeneration, brain damage induced by hypoglycemia or ischemia, or myocardial infarction [32].

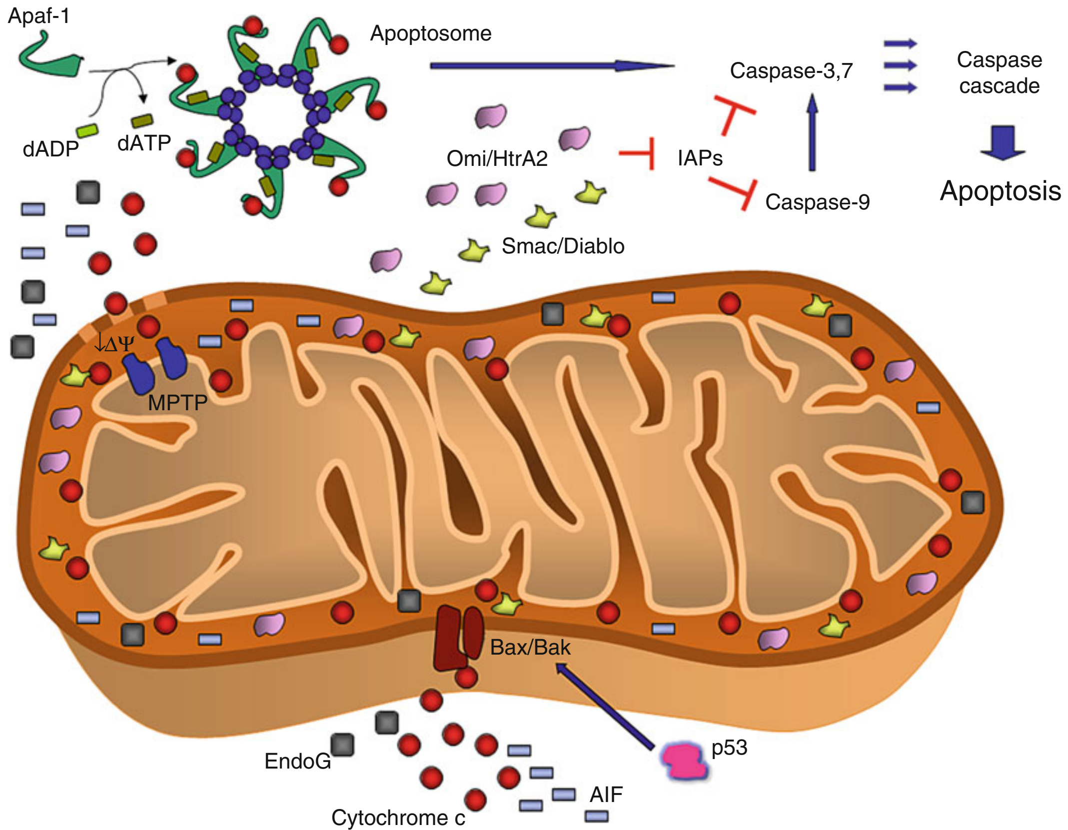

Fig. 1 Intrinsic apoptotic pathway. The intrinsic or mitochondrial apoptotic pathway, initiates with mitochondrial membrane permeabilization, which may result from p53 signaling, with formation of different pores in mitochondrial membrane, such as Bax/Bak or mitochondrial permeability transition pore (MPTP), in the latter case by leading to OMM rupture or by triggering pro-apoptotic protein translocation to mitochondria following mitochondrial depolarization. Consequently, the release of apoptotic factors to cytosol, including cytochrome c, second mitochondria derived activator of caspases/direct inhibitor-of-apoptosis binding protein with low p*I* (SMAC/Diablo), Omi serine protease (Omi/HrtA2), endonuclease G (endoG), and apoptosis-inducing factor (AIF), occurs. The released cytochrome c reacts with deoxy ATP (dATP) and apoptotic protease activating factor 1 (APAF-1), leading to apoptosome formation. The apoptosome recruits procaspase-9, activating the initiator caspase-9 and caspase cascade begins

The AIF protein is encoded by a nuclear gene, synthesized in the cytoplasm and transported to mitochondria by the general import pathway, due to the mitochondrial localization sequence (MLS) in the precursor protein. Once in the mitochondrial intermembrane space, the MLS is proteolytically cleaved, the protein refolds and incorporates flavin adenine dinucleotide (FAD) cofactor, generating the mature protein. The oxidoreductase activity, dependent of the presence of prosthetic group, is not critical for the apoptogenic effect of AIF but the structural parts of the oxidoreductase domain are necessary to the DNA biding [31].

A decrease in AIF enzymatic activity or a decrease in AIF expression results in decreased oxidative phosphorylation and increased free radicals generation [33]. On the other hand and depending on cell type and on the cell death stimuli, AIF protein is involved in programmed cell death. The basic mechanism of AIF-induced cell death consists in AIF release from the mitochondrial intermembrane space to the cytosol and then on its translocation to the nucleus. Once in the nucleus, AIF binds to DNA induces chromatin condensation and large-scale DNA fragmentation. The nuclear apoptosis induced by AIF requires a direct interaction of AIF with DNA [34]. In the cytosol, AIF also promotes a decrease in mitochondrial $\Delta\Psi$ and release of cytochrome c and further AIF release from mitochondria, promoting a positive feedback amplification loop [31, 32]. In order to be released from the mitochondrial intermembrane space, AIF must be cleaved in a specific region in order to release the protein binding to the mitochondrial inner membrane. This cleavage is performed by proteases such as calpains and cathepsins that may have access to the mitochondrial intermembrane space during apoptotic stimuli and require the presence of calcium. The truncated AIF (tAIF) is then free to execute its caspase-independent apoptotic action. An interesting aspect is the fact that binding of AIF to DNA induces large-scale DNA fragmentation, but AIF itself does not possess DNAse activity. Thus, it has been proposed that the DNA-degrading capacity of AIF could be due to the recruitment of downstream nucleases, such as cyclophilin A (CypA) [35, 36].

In this chapter, we describe the material and the methods followed by us and other authors to study the pro-apoptotic protein trafficking to and from mitochondria: immunoblotting performed with different cellular fractions and immunocytochemistry using the antibodies described in Table 1. Immunocytochemical approaches can be used to study subcellular localization of pro-apoptotic factors described in this chapter. To evaluate their mitochondrial localization, co-localization assays using antibodies constitute an effective alternative (Fig. 2). One option is to develop an immunocytochemistry using a secondary antibody conjugated with a fluorochrome such as FITC and to label mitochondria using mitochondria-selective probes such as MitoTracker Red. Another option consists in a double immunofluorescence assay using a mixture of two primary antibodies, for example one against the mitochondrial marker TOM20 and the other against the desired pro-apoptotic protein, using their respective secondary antibodies which have to be raised in different species and conjugated with two different fluorochromes (e.g., FITC-conjugated against rabbit and Texas Red-conjugated against mouse).

Table 1
List of antibodies for western blotting (WB) and immunocytochemistry (ICC)

Antibodies	MW (kDa)	Company (cat. no.)	Species cross-reactivity	Isotype	Applications (recommended dilution)
p53	53	Cell Signaling (2524)	H, M, R, Mk	Mouse IgG1	WB (1:1,000); ICC (1:250)
p53	53	Santa Cruz (sc-6243)	H, M, R	Rabbit IgG	WB (1:500); ICC (1:50)
Phospho-p53 (Ser15)	53	BioVision (3515)	H, M, R	Rabbit IgG	WB (4 μg/ml)
Phospho-p53 (Ser15)	53	Cell Signaling (9298)	H, M, R, Mk	Rabbit IgG	WB (1:1,000); ICC (1:250)
Phospho-p53 (Ser329)	53	Cell Signaling (9281)	H, M	Rabbit IgG	WB (1:1,000)
Acetyl-p53 (Lys379)	53	Cell Signaling (2570)	H, M	Rabbit IgG	WB (1:1,000)
Puma	23	Cell Signaling (4976)	H	Rabbit IgG	WB (1:1,000)
Puma	23	Cell Signaling (7467)	M, R	Rabbit IgG	WB (1:1,000)
Noxa	15	Santa Cruz (sc-56169)	H, M	Mouse IgG1	WB (1:500)
Bax	20	Cell Signaling (2772)	H, M, R, Mk	Rabbit IgG	WB (1:1,000)
Bak	25	Cell Signaling (3814)	H, M, R, Mk	Rabbit IgG	WB (1:1,000)
Bid	22 (15)	Cell Signaling (2002)	H	Rabbit IgG	WB (1:1,000)
Bid	22	Cell Signaling (2003)	M	Rabbit IgG	WB (1:1,000)
DRAM	33	Rockland (600-401-A70)	H, M, R	Rabbit IgG	WB (4 μg/ml); ICC
MDM2	90 (60)	Santa Cruz (sc-965)	H, M, R	Mouse IgG1	WB (1:500); ICC (1:50)
SOD2	25	Santa Cruz (sc-18504)	H, M, R	Goat IgG	WB (1:500); ICC (1:50)
Ubiquitin		Cell Signaling (3933)	H, M, R, Mk	Rabbit IgG	WB (1:1,000)
Bcl-2	26	Cell Signaling (2870)	H, M, R, Mk	Rabbit IgG	WB (1:1,000)

(continued)

Table 1
(continued)

Antibodies	MW (kDa)	Company (cat. no.)	Species cross-reactivity	Isotype	Applications (recommended dilution)
Bcl-xL	30	Cell Signaling (2764)	H, M, R, Mk	Rabbit IgG	WB (1:1,000); ICC (1:200)
Tom20	20	Santa Cruz (sc-11415)	H, M, R	Rabbit IgG	WB (1:500); ICC (1:100)
Cytochrome c	12	Abcam (ab13575)	M, R, H, P, Hr, Ff	Mouse IgG	WB (1:500); ICC (1:200)
Cytochrome c	12	Mitosciences (37BA11)	M, R, C, H, Ce	Mouse IgG	WB (0.5 μg/ml); ICC (1 μg/ml)
Smac/Diablo	21	Cell Signaling (2954)	H, Mk	Mouse IgG	WB (1:1,000); ICC (1:100)
Apaf-1	130	Millipore (AB16503)	H, R, M	Rabbit IgG	WB (1:1,000)
Omi	48	Abcam (ab33041)	M, H	Mouse IgG	ELISA; WB (1:1,000)
AIF	57	Santa Cruz (sc-13116)	H, R, M	Mouse gG2b	WB (1:1,000); ICC (1:100)
Anti-rabbit IgG-AP		Santa Cruz (sc-2007)		Goat IgG	WB (1:5,000)
Anti-goat IgG-AP		Santa Cruz (sc-2022)		Donkey IgG	WB (1:5,000)
Anti-mouse IgG1-AP		Santa Cruz (sc-2066)		Goat IgG	WB (1:5,000)
Anti-rabbit IgG-FITC		Santa Cruz (sc-2012)		Goat IgG	ICC (1:400)
Anti-mouse IgG-TR		Santa Cruz (sc-2781)		Goat IgG	ICC (1:400)

Ce Caenorhabditis elegans, *C* cow, *Ff* fruit fly, *Hr* horse, *H* human, *Mk* monkey, *M* mouse, *P* pigeon, *R* rat

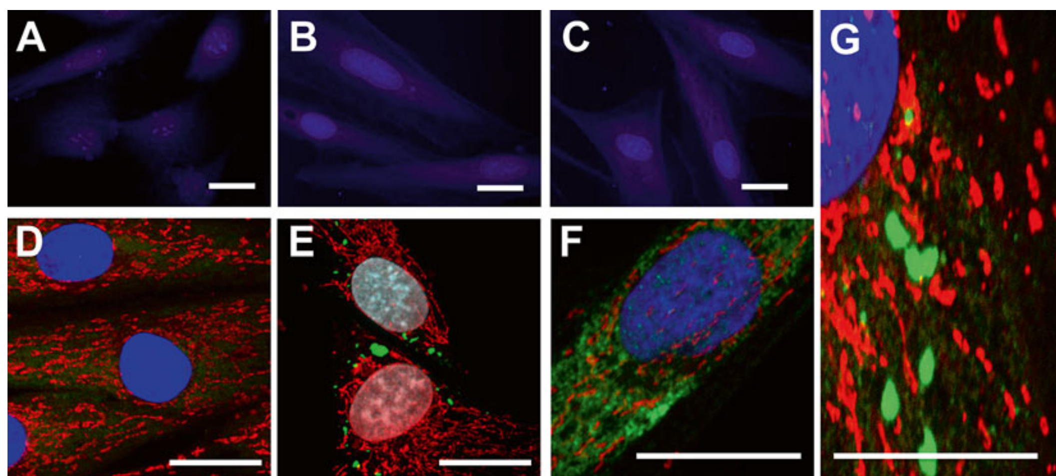

Fig. 2 Representative micrographs of fluorescence microscopy of immunocytochemistry-labeled apoptotic proteins. Images show H9c2 cardiomyoblasts treated with the anticancer agent Doxorubicin (DOX) and imaged for p53 (**a**–**c**) and Bax (**d**–**g**). Panels **a**–**c** show H9c2 cells treated with vehicle (**a**), 0.5 μM (**b**) and 1 μM (**c**) DOX for 24 h. After treatment, H9c2 cells were fixed with ice cold methanol (which removed nuclear bound DOX), labeled with an antibody against p53. Epifluorescence microscopy images in panels **a**–**c** illustrate increased p53 nuclear labeling (in *blue*) after H9c2 cells treatment with DOX. Cells were observed by epifluorescence microscopy using a Nikon Eclipse TE2000U microscope equipped with a 40× Plan Fluor 1.3 NA oil immersion DIC objective and images were processed using Metamorph software (Universal Imaging, Downingtoen, PA). Panels **d**–**g** show confocal microscopy images of H9c2 cells labeled with Hoechst 33342 (nucleus, *blue* and *pink*), Mitotracker Red (mitochondria, *red*) and Bax (*green*). Confocal microscopy of H9c2 cells treated with 0 (**d**), 0.5 μM (**e**, **g**) and 1 μM (**f**) DOX for 24 h. After treatment, cells were fixed in paraformaldehyde (in order to maintain the integrity of the mitochondrial network) and subsequently immunolabeled with an antibody against Bax. Increased immunolabeling for Bax is observed after DOX treatment, especially forming large clusters in the cytosol of treated cells. This is clearly visible in panel **g**, obtained with a higher magnification. Images in panels **d**–**g** were obtained by using a Nikon C-1 laser scanning confocal microscope equipped with a 60× Plan Apo 1.4 NA oil immersion DIC objective. Images were captured using the Nikon EZ-C1 software (version 2.01). *White bar* in all panels represents 20 μm

2 Materials

2.1 Nuclear, Mitochondrial, and Cytosolic Fraction Isolation from Cultured Cells

1. PBS: 137.93 mM NaCl, 2.67 mM KCl, 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4.
2. Buffer A: 250 mM Sucrose, 20 mM Hepes, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, pH 7.5 (adjusted with KOH).
3. Buffer B: 250 mM Sucrose, 10 mM $MgCl_2$.
4. Buffer C: 350 mM Sucrose, 0.5 mM $MgCl_2$.
5. Nuclear buffer: 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26 % glycerol (v/v), pH 7.9.

2.2 Western Blotting

1. Protein quantification: Bradford (B6916; Sigma, St. Quentin Fallavier, France) or bicinchoninic acid (BCA) (23227; Thermo Scientific, Rockford, IL, USA) assays.
2. Laemmli buffer: 62.5 mM Tris–HCl at pH 6.8, 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue. Add 5 % β-mercaptoethanol prior to use (*see* **Note 1**).
3. Mini-PROTEAN system with Mini Trans-Blot module, gel cassettes and casting stand, short and spacer plates, combs, external power supply (Bio-Rad, Hercules, CA, USA) and rollers or shakers for incubations.
4. Prestained protein standard (161-0374; Bio-Rad), polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) and Ponceau S staining solution: 0.1 % (w/v) in 5 % acetic acid.
5. Buffers: Running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3); Transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) and TBS-T (10 mM Tris–HCl at pH 8.0, 150 mM NaCl, 0.1 % Tween-20).
6. ECF substrate (RPN5785; GE Healthcare, Munich, Germany) and a detection system like VersaDoc (Bio-Rad).

2.3 Immunoprecipitation

1. Protein G PLUS-Agarose (sc-2002, Santa Cruz Biotechnology, Santa Cruz, CA, USA).
2. IP buffer I (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 % Na-deoxycholate, 1 % NP-40, 1 mM Na_3VO_4).
3. IP buffer II (50 mM Tris–HCl, pH 7.4, 75 mM NaCl, 0.1 % Na-deoxycholate, 0.1 % NP-40, 1 mM Na_3VO_4).
4. IP buffer III (50 mM Tris–HCl, pH 7.4, 0.05 % Na-deoxycholate, 0.05 % NP-40, 1 mM Na_3VO_4).
5. Laemmli buffer: 62.5 mM Tris–HCl at pH 6.8, 25 % glycerol, 2 % SDS, 0.01 % bromophenol blue. Add 5 % β-mercaptoethanol prior to use.

2.4 Immunocytochemistry

1. Sterile glass coverslips, 6-well plates, lancets, forceps, microscope slides and a dark humidified chamber.
2. Culture medium and mitochondrial fluorescent probes: MitoTracker Red CMXRos (M7512; Invitrogen, Paisley, UK) or MitoTracker Green FM (M7514; Invitrogen).
3. PBS: 137 mM NaCl in 10 mM phosphate buffer, pH 7.4.
4. 4 % Formaldehyde in PBS, 0.2 % Triton X-100 in PBS and 1 % BSA in PBS.
5. Prolong Gold antifade medium with DAPI (P36935; Invitrogen) or without DAPI (P36934; Invitrogen) and nail polish.

3 Methods

3.1 Nuclear, Mitochondrial, and Cytosolic Fraction Isolation from Cells in Culture

1. Supplement buffer A with 1 μg/ml of leupeptin, antipain, chymostatin, and pepstatin A, 1 mM of DTT and 100 μM of PMSF. Supplement buffer B and C with 1 mM of DTT and 100 μM of PMSF. Supplement the nuclear buffer with 300 mM of NaCl (high salt helps lyse nuclear membranes and forces DNA into solution).
2. Grow cells on cell culture dishes in an appropriated cell culture medium and perform the desired treatment (*see* **Note 2**).
3. After treatment, aspirate or collect (if you are interested in the floating cells) the incubation media. Rinse the adherent cells with 5 ml of PBS and waste it.
4. Harvest adherent cells with 3–5 ml of trypsin. Inhibit the trypsin with 3–5 ml of growth media containing FBS (*see* **Note 3**).
5. Centrifuge between 300 and 400 × *g* for 5 min at 4 °C in order to collect all cells without damaging membrane integrity.
6. Aspirate the supernatant and rinse the cell pellet with 2 ml of PBS. Perform again the centrifugation step, discard the supernatant and resuspend the pellet in 1 ml of complete buffer A. Incubate for 15 min on ice.
7. Homogenize cells in a Potter-Elvehjem homogenizer with a Teflon pestle (30–40 strokes), or alternatively pass cell suspension through a 25 G needle ten times using a 1 ml syringe. This procedure should also be performed on ice.
8. Centrifuge the cellular suspension at 720 × *g* for 5 min at 4 °C. Remove the supernatant (containing mitochondrial and cytosolic fractions) and keep it on ice.
9. Resuspend the nuclear pellet again in 1 ml of complete buffer A. Homogenize the pellet again in a Potter–Elvehjem homogenizer with a Teflon pestle, or alternatively pass through a 25 G needle. Centrifuge again at 720 × *g* for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 0.5 ml of complete buffer B and pour the nuclear suspension on the complete buffer C. Centrifuge at 1,430 × *g* for 5 min at 4 °C. Remove the supernatant and discard it. Resuspend the nuclear pellet in 50 μl of nuclear buffer supplemented with 300 mM of NaCl. Homogenize the nuclear pellet passing nuclear suspension through a 27 G needle ten times using a 0.5 ml syringe. Store at −80 °C until further analysis.
10. Centrifuge the supernatant collected in **step 8** at 14,000 × *g* for 10 min at 4 °C. Resuspend the pellet (mitochondrial fraction) in 50 μl of buffer 1 and store at −80 °C until further analysis.
11. Collect the supernatant (containing the cytosolic fraction) and centrifuge in an ultracentrifuge at 100,000 × *g* for 30 min at

4 °C. Discard the pellet (containing the membrane fractions) and concentrate the supernatant (containing the cytosolic fraction) by lyophilization or by tangential flow filtration. Store at −80 °C until further analysis be performed (*see* **Note 4**).

In Fig. 3, a flowchart outlining the isolation procedure is presented

3.2 Western Blotting

3.2.1 SDS-PAGE

1. Determine protein content of all fractions by standard procedures such as the Bradford or the BCA method using 5 μl of aliquot.
2. Mix the samples with the appropriated volume of Laemmli buffer (1:1).
3. Prepare the Sodium Dodecyl Sulfate (SDS) 10 % polyacrylamide resolving gel (*see* **Note 5**) by mixing 2.5 ml Tris–HCl 1.5 M pH 8.8, 0.1 ml SDS 10 %, 50 μl ammonium persulfate 10 %, 2.5 ml acrylamide/bisacrylamide (29:1) 40 %, 4.9 ml of distilled water (for a 0.75–1.0 mm thick gel). Add 5 μl of TEMED to initiate polymerization. Cast gel within an assembled gel cassette allowing space for stacking gel, gently overlay with water and wait until polymerization.
4. Prepare the stacking gel (4 % polyacrylamide) by mixing 1.25 ml Tris–HCl 0.5 M, pH 6.8, 50 μl SDS 10 %, 25 μl ammonium persulfate 10 %, 0.5 ml acrylamide–bisacrylamide (29:1) 40 %, 3.2 ml of distilled water, and 5 μl of TEMED. Insert a gel comb immediately and wait until polymerization.
5. Denature the samples by boiling at 95–100 °C for 5 min.
6. For polyacrylamide gel electrophoresis, we normally use Mini-PROTEAN systems from Bio-Rad, but other alternative systems can be used. After complete polymerization, place the gel into the electrophoretic chamber with running buffer, load the volume of sample corresponding to 5–25 μg of protein in each individual well (*see* **Note 6**), as well as 6 μl of prestained protein standard (e.g., Precision Plus Protein Dual Color Standards from Bio-Rad) into one of the other lanes.
7. Run electrophoresis at constant voltage (100–120 V). Running can be monitored by observing migration of prestained protein standards and bromophenol blue front. Stop running when the bromophenol blue band leaves the lower end of the gel. Keep gels in running buffer until ready to transfer.

3.2.2 Wet/Tank Electrophoretic Transfer

1. Activate polyvinylidene difluoride (PVDF) membrane in methanol for 15 s. Transfer the membrane from methanol to transfer buffer and incubate on shaker for at least 5 min. Soak also pads, filter papers, and the gel in transfer buffer before use.
2. Assemble the transfer sandwich in a shallow tray filled with transfer buffer as follows: Black side of the sandwich (cathode),

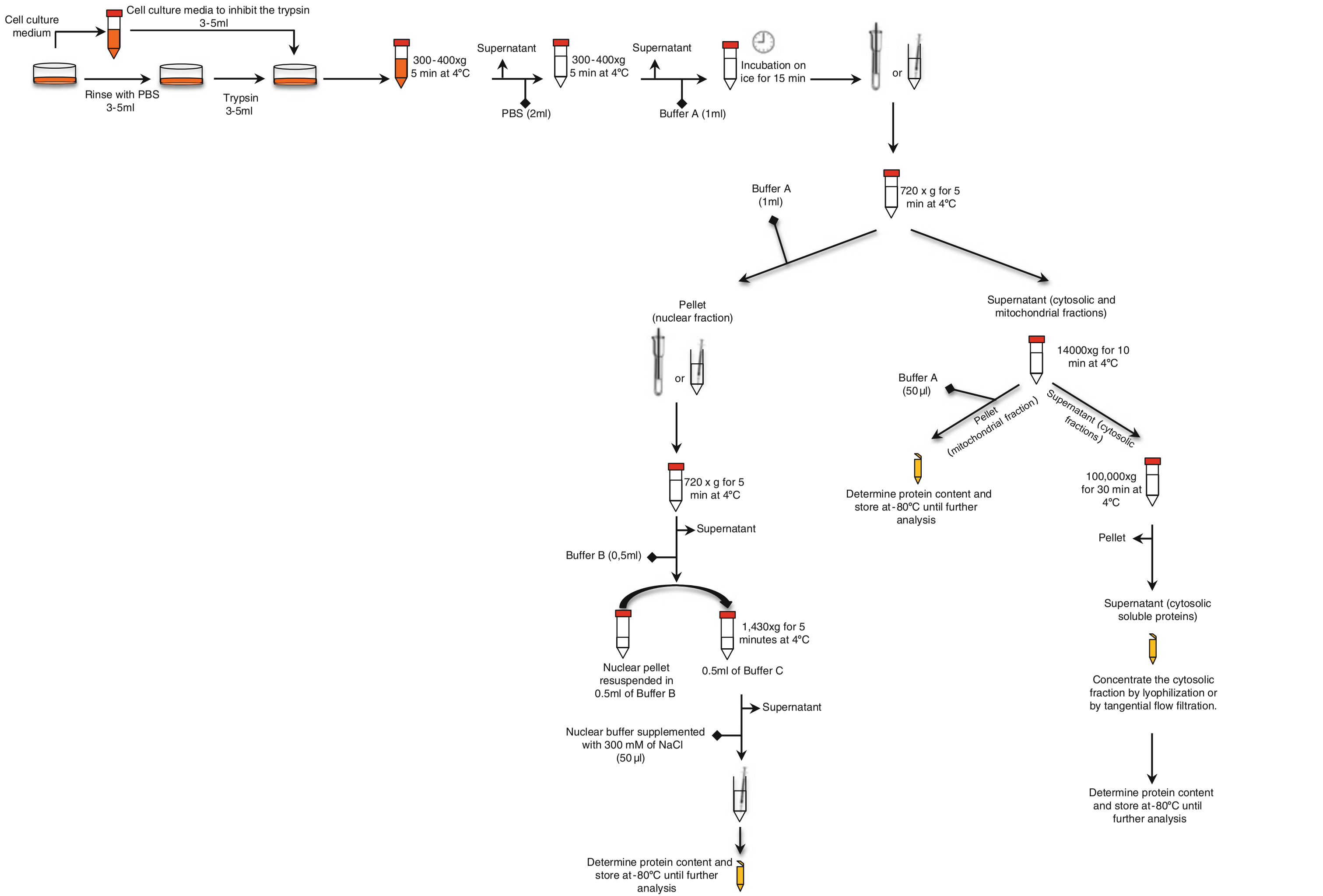

Fig. 3 Flowchart outlining the isolation of nuclear, mitochondrial, and cytosolic fractions from cells in culture. All the material was kept on ice during all the procedures and all the centrifugations were performed at 4 °C

soaked pads, filter paper, gel, PVDF membrane, filter paper, soaked pad, and white side of the sandwich (anode). Avoid bubbles formation between gel and PVDF membrane. Fill tank apparatus with transfer buffer and run at 350 mA for 90 min with an ice pack or another cooling system (*see* **Note** 7).

3. Stain the membrane with Ponceau S for 5 min to check protein transfer. Finally, transfer the membrane to TBS-T to rinse Ponceau staining.

3.2.3 Enzymatic Immunodetection

1. Block the membrane to reduce nonspecific binding with 5 % skim milk in TBS-T overnight at 4 °C or alternatively for 2 h at room temperature (*see* **Notes 8** and **9**).
2. Incubate the membrane with the primary antibody at the recommended dilution (*see* Table 1) in 1 % skim milk in TBT-T for 4 h at 4 °C or overnight.
3. Wash three times with TBS-T for 5 min at room temperature.
4. Incubate the membrane with the corresponding alkaline phosphatase conjugated secondary antibody (1:5,000) in 1 % skim milk in TBT-T for 2 h at 4 °C.
5. Wash three times with TBS-T for 20 min at room temperature.
6. Detect the immunoconjugates using the western blotting ECF substrate (or similar) according to the manufacturer's protocol. Use fluorescence scanning equipment such as VersaDoc (Bio-Rad) to develop the image and analyze it using the software QuantityOne (Bio-Rad) or Image J (or similar). Please notice that local vs. global background subtraction must be chosen since this will affect the final results.

3.3 Immunoprecipitation

P53 ubiquitination and the physical interaction of p53 with some related proteins such as Bcl-2, Bcl-xL, Bax, and SOD-2 can be evaluated by p53 immunoprecipitation from homogenates and subsequent immunoblot analysis with antibodies against these related proteins.

1. Incubate a volume of extracts corresponding to equal quantity of protein (e.g., 300 μg of protein) with 5 μl mouse anti-p53 (2524; Cell Signaling Technology) for 1 h at 4 °C under orbital shaking conditions.
2. Add 20 μl of protein G PLUS-Agarose and incubate overnight at 4 °C under orbital shaking conditions.
3. Centrifuge 1 min at 16,000 × *g* and discard the supernatant fraction.
4. Add 1 ml of IP buffer I to the pellet and mix the sample on an oscillatory shaker for 20 min at 4 °C.
5. Centrifuge for 1 min at 16,000 × *g* and discard the supernatant fraction.

6. Repeat with IP buffer II.
7. Repeat with IP buffer III.
8. Centrifuge 1 min at 16,000 × *g* and discard the supernatant fraction.
9. Add 20 μl of Laemmli buffer and boil for 5 min.
10. Centrifuge again 1 min at 16,000 × *g* to pellet the agarose beads.
11. Transfer the supernatant fraction to a new tube, subject it to SDS-PAGE and immunoblot analysis as previously described (in Subheading 3.2) using primary antibodies against p53 (sc-6243; Santa Cruz Biotechnology), BCL-2, BCL-xL, BAX, SOD-2 and/or ubiquitin (check Table 1 for references and recommended dilutions).

3.4 Immunocyto-chemistry

3.4.1 Cell Seeding for Morphological Studies

1. Seed cells on glass coverslips in 6-well plates and wait for 24 h for cell adhesion at 37 °C in a 5 % CO_2 atmosphere.

3.4.2 Staining with Mitochondrial Specific Fluorescent Dye (Optional)

1. Remove culture medium and incubate cells with 125 nM MitoTracker Red CMXRos (M7512; Invitrogen) in culture medium for 20 min at 37 °C. Alternatively, 0.5 μM green-fluorescing MitoTracker Green FM (M7514; Invitrogen) for 30 min at 37 °C can be used to label mitochondria (*see* **Note 10**).
2. Replace staining solution with fresh pre-warmed media and subject cells to subsequent processing steps.

3.4.3 Fixation and Permeabilization

1. Remove the incubation media and fix cells in 4 % formaldehyde in PBS for 15 min at 37 °C.
2. Rinse three times with PBS for 5 min each.
3. Permeabilize cells with 0.2 % Triton X-100 in PBS for 10 min.
4. Rinse three times with PBS for 5 min each.

3.4.4 Immunolabeling

1. Block to reduce nonspecific binding with 1 % BSA in PBS for 1 h at 4 °C (*see* **Note 11**).
2. Distribute 100–150 μl of primary antibody (or mixture of antibodies) at 1:250 in PBS, 1 % BSA on each coverslip and incubate 90 min in a humidified chamber at room temperature.
3. Remove the primary antibody (it can be stored in 0.05 % sodium azide) and rinse three times with PBS for 5 min.
4. Incubate with the corresponding fluorescence-conjugated secondary antibody (or mixture of antibodies) at 1:400 in PBS, 1 % BSA for 1 h in a dark humidified chamber at 37 °C.
5. Remove the secondary antibody and rinse with PBS three times for 5 min each with PBS.

3.4.5 Mounting Coverslips

1. Mount coverslips on glass slides using Prolong Gold antifade medium (P36934; Invitrogen). If nuclear labeling is desired, use Prolong Gold antifade medium with DAPI (P36935; Invitrogen).
2. Allow to dry overnight, seal with nail polish, and store at −80 °C until analysis under a confocal microscope (LSM 510Meta; Zeiss).

4 Notes

1. Alternatively to the use of β-mercaptoethanol in Western Blot sample preparation, dithiothreitol (DTT) can be used in the same proportion.
2. In order to have enough protein for the different cellular fractions, it is often necessary to start with large amount of cells, especially for large volume cells. Optimization process for cell density is required.
3. If the incubation medium is saved in order to collect floating cells, it can be used to inhibit the trypsin.
4. It is advisable to test the purity of each sample using specific antibodies for each fraction. For example, one strategy involves cross-labeling samples from different fractions with antibodies against a particular histone (nuclear marker), the voltage-dependent anion channel (VDAC) or TOM20 (mitochondrial markers), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, a cytosolic marker).
5. Choose the percentage of the gel to be used according to the molecular weight of proteins of interest. In general: 4–5 % gels: >250 kDa; 7.5 % gels: 250–120 kDa; 10 % gels: 120–40 kDa; 13 % gels: 40–15 kDa; 15 % gels: <20 kDa.
6. Apply 5–25 μg total protein of sample to each well of a 0.75–1.0 mm thick gel. For thicker gels (1.5 mm thick), apply up to 25–40 μg in each well.
7. For proteins smaller than 20 kDa, transfer proteins from gel to PVDF membrane at 350 mA for 1 h in transfer buffer. For proteins larger than 120 kDa, transfer to PVDF membrane at 350 mA for 140 min in transfer buffer supplemented with 0.05 % SDS.
8. Some antibodies require the use of BSA (1–5 %) as a blocking agent. Read the vendor's instructions before following the method.
9. All incubation and washing steps are carried out while gently shaking.

10. Alternatively, regular medium without phenol red or Krebs medium can be used, to avoid interferences with the probe's fluorescence.
11. Unspecific binding of the antibodies can be also blocked with 10 % serum from the species in which the secondary antibody was raised.

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References

1. Galluzzi L, Kepp O, Trojel-Hansen C et al (2012) Mitochondrial control of cellular life, stress, and death. Circ Res 111:1198–1207
2. Chao DT, Korsmeyer SJ (1998) BCL-2 family: regulators of cell death. Annu Rev Immunol 16:395–419
3. Reed JC, Zha H, Aime-Sempe C et al (1996) Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 406:99–112
4. Scorrano L, Oakes SA, Opferman JT et al (2003) BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science 300:135–139
5. Bernardi P (2013) The mitochondrial permeability transition pore: a mystery solved? Front Physiol 4:95
6. Precht TA, Phelps RA, Linseman DA et al (2005) The permeability transition pore triggers Bax translocation to mitochondria during neuronal apoptosis. Cell Death Differ 12:255–265
7. Liu B, Chen Y, St Clair DK (2008) ROS and p53: a versatile partnership. Free Radic Biol Med 44:1529–1535
8. Vazquez A, Bond EE, Levine AJ et al (2008) The genetics of the p53 pathway, apoptosis and cancer therapy. Nat Rev Drug Discov 7:979–987
9. Galluzzi L, Morselli E, Kepp O et al (2011) Mitochondrial liaisons of p53. Antioxid Redox Signal 15:1691–1714
10. Liu Y, Kulesz-Martin M (2001) p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis 22:851–860
11. Laptenko O, Prives C (2006) Transcriptional regulation by p53: one protein, many possibilities. Cell Death Differ 13:951–961
12. Yamaguchi H, Woods NT, Piluso LG et al (2009) p53 acetylation is crucial for its transcription-independent proapoptotic functions. J Biol Chem 284:11171–11183
13. Shieh SY, Ikeda M, Taya Y et al (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. Cell 91:325–334
14. Cox ML, Meek DW (2010) Phosphorylation of serine 392 in p53 is a common and integral event during p53 induction by diverse stimuli. Cell Signal 22:564–571
15. Yap DB, Hsieh JK, Zhong S et al (2004) Ser392 phosphorylation regulates the oncogenic

function of mutant p53. Cancer Res 64: 4749–4754

16. Hu W, Feng Z, Levine AJ (2012) The regulation of multiple p53 stress responses is mediated through MDM2. Genes Cancer 3: 199–208
17. Tasdemir E, Maiuri MC, Galluzzi L et al (2008) Regulation of autophagy by cytoplasmic p53. Nat Cell Biol 10:676–687
18. Vega-Naredo I, Caballero B, Sierra V et al (2009) Sexual dimorphism of autophagy in Syrian hamster Harderian gland culminates in a holocrine secretion in female glands. Autophagy 5:1004–1017
19. Yamamoto H, Ozaki T, Nakanishi M et al (2007) Oxidative stress induces p53-dependent apoptosis in hepatoblastoma cell through its nuclear translocation. Genes Cells 12:461–471
20. Salminen A, Kaarniranta K (2009) SIRT1: regulation of longevity via autophagy. Cell Signal 21:1356–1360
21. Chipuk JE, Kuwana T, Bouchier-Hayes L et al (2004) Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303:1010–1014
22. Sardao VA, Oliveira PJ, Holy J et al (2009) Doxorubicin-induced mitochondrial dysfunction is secondary to nuclear p53 activation in H9c2 cardiomyoblasts. Cancer Chemother Pharmacol 64:811–827
23. Moll UM, Wolff S, Speidel D et al (2005) Transcription-independent pro-apoptotic functions of p53. Curr Opin Cell Biol 17:631–636
24. Mihara M, Erster S, Zaika A et al (2003) p53 has a direct apoptogenic role at the mitochondria. Mol Cell 11:577–590
25. Zhao Y, Chaiswing L, Velez JM et al (2005) p53 translocation to mitochondria precedes its nuclear translocation and targets mitochondrial oxidative defense protein-manganese superoxide dismutase. Cancer Res 65: 3745–3750
26. Green ML, Pisano MM, Prough RA et al (2013) Release of targeted p53 from the mitochondrion as an early signal during mitochondrial dysfunction. Cell Signal 25: 2383–2390
27. Goldstein JC, Munoz-Pinedo C, Ricci JE et al (2005) Cytochrome c is released in a single step during apoptosis. Cell Death Differ 12: 453–462
28. Martinez-Ruiz G, Maldonado V, Ceballos-Cancino G et al (2008) Role of Smac/DIABLO in cancer progression. J Exp Clin Cancer Res 27:48
29. Kaufmann T, Strasser A, Jost PJ (2012) Fas death receptor signalling: roles of Bid and XIAP. Cell Death Differ 19:42–50
30. Kilbride SM, Prehn JH (2013) Central roles of apoptotic proteins in mitochondrial function. Oncogene 32:2703–2711
31. Susin SA, Lorenzo HK, Zamzami N et al (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397:441–446
32. Cande C, Cohen I, Daugas E et al (2002) Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84:215–222
33. Lipton SA, Bossy-Wetzel E (2002) Dueling activities of AIF in cell death versus survival: DNA binding and redox activity. Cell 111: 147–150
34. Ye H, Cande C, Stephanou NC et al (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. Nat Struct Biol 9:680–684
35. Baritaud M, Boujrad H, Lorenzo HK et al (2010) Histone H2AX: the missing link in AIF-mediated caspase-independent programmed necrosis. Cell Cycle 9:3166–3173
36. Cande C, Vahsen N, Kouranti I et al (2004) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. Oncogene 23: 1514–1521