

# Rapid induction of mitochondrial events and caspase-independent apoptosis in Survivin-targeted melanoma cells

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The inhibitor of apoptosis (IAP) protein Survivin is expressed in most cancers and is a key factor in maintaining apoptosis resistance. Although several IAPs have been shown to act as direct inhibitors of caspases, the precise antiapoptotic function of Survivin remains controversial. To clarify the mechanism by which Survivin protects cells, we investigated the kinetics of apoptosis and apoptotic events following Survivin inhibition utilizing a melanoma cell line harboring a tetracycline-regulated Survivin dominant-negative mutant (Survivin-T34A). Blocking Survivin resulted in both caspase activation and apoptosis; however, the level of apoptosis was only partially reduced by caspase inhibition. Survivin blockade also resulted in mitochondrial events that preceded caspase activation, including depolarization and release of cytochrome *c* and Smac/DIABLO. Levels of other IAPs were not altered in Survivin-targeted cells, although modest cleavage of XIAP and Livin was observed. The earliest proapoptotic event observed in Survivin-targeted cells was nuclear translocation of mitochondrial apoptosis-inducing factor (AIF), known to trigger both apoptotic mitochondrial events and caspase-independent DNA fragmentation. These findings suggest that a key anti-apoptotic function of Survivin relates to inhibition of mitochondrial and AIF-dependent apoptotic pathways, and its expression in melanoma and other cancers likely protects against both caspase-independent and -dependent apoptosis.

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## Introduction

Apoptosis, or programmed cell death, is a physiologic means of eliminating normal cells and is commonly dysregulated in cancer (Reed, 1999). Resistance to apoptosis may result from failure to develop a proteolytic caspase cascade from either the 'extrinsic' pathway, initiated by activation of membrane-bound

death receptors leading to cleavage of caspase-8 (Ashkenazi and Dixit, 1998), or the 'intrinsic' pathway characterized by mitochondrial depolarization, release of cytochrome *c*, and subsequent activation of caspase-9 (Green and Reed, 1998). In some cells, these two pathways can be linked by caspase-8-mediated cleavage of the Bcl-2 family member Bid, which leads to activation of caspase-9 (Green, 1998). Either caspase-8 or -9 can activate terminal caspase-3 and -7, which cleave proteins involved in cytoskeletal and nuclear structure (Thornberry and Lazebnik, 1998), resulting in the cell shrinkage and DNA fragmentation of late-stage apoptosis (Kerr *et al.*, 1972). Prior to these gross morphologic changes, there is transposition of phosphatidylserine to the outer plasma membrane leaflet that represents the earliest detectable morphological event in cells undergoing apoptosis (Reutelingsperger and van Heerde, 1997). Although caspase activation is considered a hallmark of apoptotic cell death, other less-defined cell death pathways have been described that appear not to require caspase activation (Borner and Monney, 1999; Sperandio *et al.*, 2000). In particular, some apoptotic stimuli activate apoptosis-inducing factor (AIF), a mitochondrial flavoprotein that induces cytochrome *c* release and subsequent caspase activation; AIF also translocates to the nucleus and causes nuclear fragmentation that is not blocked by caspase inhibitors (Susin *et al.*, 1999; Daugas *et al.*, 2000). While deficiencies at multiple levels of both the extrinsic and intrinsic pathways have been found in cancer cells, acquired defects relating to the AIF-dependent pathway have not been described (Reed, 2003).

The inhibitor of apoptosis (IAP) proteins are conserved from insects to humans and serve as key regulators of caspase activity (Deveraux *et al.*, 1997). The IAPs identified in humans include cIAP-1, cIAP-2, NIAP, XIAP, Livin/ML-IAP, and Survivin, all of which possess one or more cysteine/histidine-rich zinc-binding (baculovirus IAP repeat, BIR) domains (Reed, 2000; Kasof and Gomes, 2001). The IAPs are thought to protect cells against apoptosis by acting as caspase inhibitors (Deveraux and Reed, 1999). The BIR1 and BIR2 domains of cIAP-1 and cIAP-2 (Roy *et al.*, 1997), NIAP (Maier *et al.*, 2002), and XIAP (Deveraux *et al.*, 1997) have all been shown to inhibit downstream caspase-3 and -7. The cIAP-1 and cIAP-2 proteins and XIAP have also been shown to block activation of

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upstream caspase-9 (Deveraux *et al.*, 1998, 1999). Livin contains a single BIR domain, and binding studies demonstrated specific interactions with caspase-9, -3, and -7 (Kasof and Gomes, 2001). IAP proteins are regulated by interaction with the mitochondrial protein Smac/DIABLO, which may be released into the cytosol upon apoptotic stimulation and through IAP sequestration results in elevated caspase activity (Du *et al.*, 2000; Verhagen *et al.*, 2000). Some IAP proteins are also regulated by proteolysis via the ubiquitin–proteasome pathway (Yang *et al.*, 2000), and caspase-dependent cleavage of XIAP has been described in cells stimulated with anti-Fas (Deveraux *et al.*, 1999).

Survivin, like Livin, also contains a single BIR domain, but is distinguished among the IAPs by its ubiquitous expression during development and absence in most normal tissues (Ambrosini *et al.*, 1997; Adida *et al.*, 1998). Survivin is re-expressed in most cancers (Velculescu *et al.*, 1999) and associated with tumor aggression and decreased patient survival (Altieri *et al.*, 1999), making it an attractive diagnostic and therapeutic target (Altieri, 2001). Crystallographic studies revealed a dimeric structure, symmetrically oriented around an intermolecular zinc atom, with an extensive hydrophobic interface involving the BIR domains of each molecule (Chantalat *et al.*, 2000; Verdecia *et al.*, 2000). BIR mutations interfering with dimerization compromise antiapoptotic activity and result in ‘dominant-negative’ mutants (Muchmore *et al.*, 2000), which we and others have employed to block endogenous Survivin and precipitate apoptosis in transformed cells (Grossman *et al.*, 1999a, b, 2001b; Li *et al.*, 1999; Mesri *et al.*, 2001). Survivin protects cells against cytotoxic drugs (Li *et al.*, 1998) and ultraviolet-B (UVB) radiation (Grossman *et al.*, 2001a) but not anti-Fas (Grossman *et al.*, 2001a), suggesting that it predominantly functions as a regulator of intrinsic rather than extrinsic apoptotic pathways. The presumption has been that Survivin, like other IAPs, protects cells from apoptosis through caspase inhibition (Reed, 2001). Consistent with this notion, a physical interaction of Survivin with caspase-9 has been demonstrated (O’Connor *et al.*, 2000), and activation of both caspase-9 (O’Connor *et al.*, 2000; Mesri *et al.*, 2001) and caspase-3 (Li *et al.*, 1998; Mesri *et al.*, 2001) were observed in Survivin-targeted cells.

Recent data, however, have caused some confusion as to the general mechanism by which Survivin protects cells from apoptosis. Conflicting reports of the capacity of Survivin to bind caspase-3 (Banks *et al.*, 2000; Conway *et al.*, 2000; Shin *et al.*, 2001), and the demonstration in Survivin-targeted cells of mitochondrial content release (Mesri *et al.*, 2001; Shankar *et al.*, 2001), generally thought to be upstream of caspase activation in apoptotic pathways (Reed, 2000), have raised the question of whether caspase inhibition is indeed the primary antiapoptotic function of Survivin. Here, we used an inducible Survivin BIR mutant to determine the chronology of apoptotic events following inhibition of Survivin in melanoma cells. We report that the first detectable effect of Survivin targeting is mitochondrial AIF translocation, which results in

mitochondrial events that precede caspase activation and promotes caspase-independent DNA fragmentation.

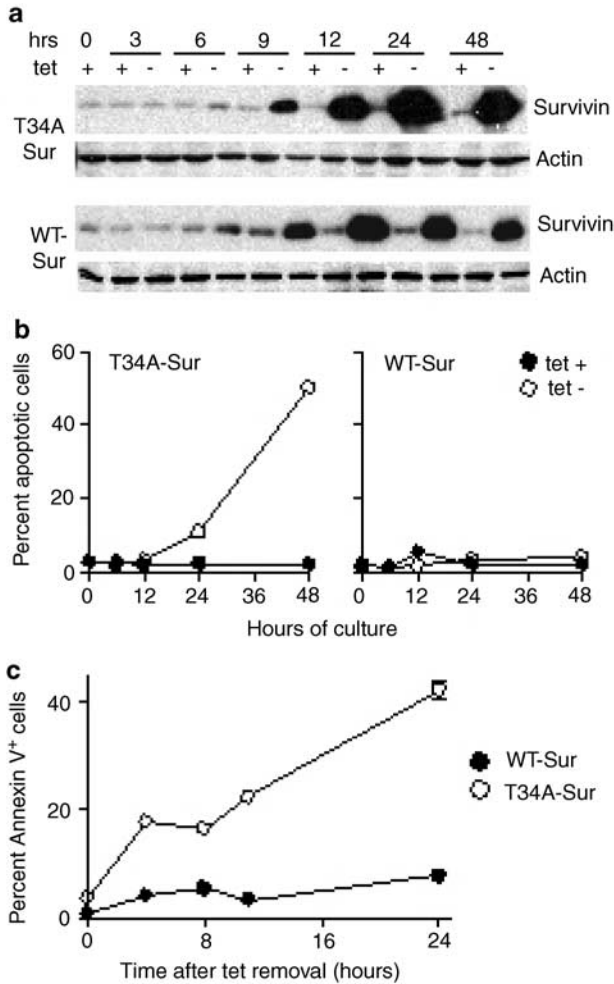
## Results

### *Kinetics of Survivin targeting and apoptosis*

To examine the kinetics of apoptosis following interference with endogenous Survivin function, we utilized a melanoma cell line stably transfected with a tetracycline (tet)-regulated Survivin Thr<sup>34</sup>→Ala mutant (T34A-Sur). A similarly prepared cell line with tet-regulated expression of wild-type Survivin (WT-Sur) served as a control. We assessed transgene expression and apoptosis induction in these cells over a 48-h period after removal of tet from the culture medium. Tet-regulated expression of both Survivin-T34A and wild-type Survivin in T34A-Sur and WT-Sur cells, respectively, could be detected over endogenous Survivin by 6 h with maximal expression between 12 and 24 h (Figure 1a). Upon over-exposure, blots revealed low-level expression by 2–4 h (not shown). Corresponding blots for  $\beta$ -actin confirmed equivalent levels of protein for each lane (Figure 1a). Tet-regulated apoptosis, as determined by DNA content analysis, was seen in T34A-Sur cells by 24 h, and was maximal by 48 h after mutant induction (Figure 1b). By contrast, tet-regulated induction of wild-type Survivin in WT-Sur cells was not associated with apoptosis even at 48 h (Figure 1b). To define more precisely the kinetics of apoptosis induced by tet-regulated Survivin targeting, we next measured Annexin V binding to the outer membrane phosphatidylserine, an early marker of apoptosis (Reutelingsperger and van Heerde, 1997). Cells were cultured in the absence of tet, and then stained with FITC-conjugated Annexin V over a 24-h period. As shown in Figure 1c, Annexin V binding could be detected in T34A-Sur cells as early as 4 h, and by 24 h identified 40% of the cells, approaching the percent of cells ultimately undergoing apoptosis by 48 h (Figure 1b). By contrast, positive Annexin V staining was not seen in tet-deprived WT-Sur cells even at 24 h (Figure 1c). Thus, apoptosis is rapidly induced by Survivin targeting, with detection of early apoptotic cells essentially coinciding with the onset of detectable Survivin-T34A expression.

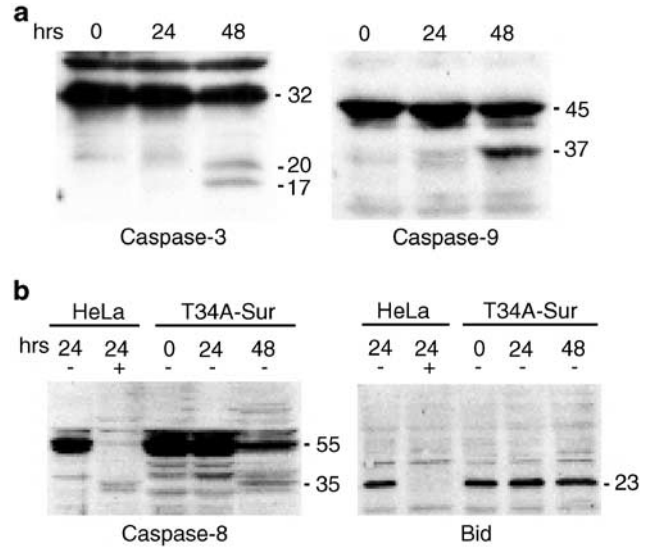
### *Survivin targeting causes both caspase-dependent and -independent apoptosis*

We next examined activation of individual caspases associated with tet-regulated Survivin targeting in T34A-Sur cells. The terminal caspase-3 was activated by 48 h as evidenced by a decrease in the 32 kDa precursor and appearance of 20 and 17 kDa cleavage fragments (Figure 2a). We suspected that caspase-3 activation in this system could be triggered by the upstream initiator caspase-9, given the reported binding and inhibition by Survivin of caspase-9 (O’Connor *et al.*, 2000). Consistent with this notion, Survivin targeting



**Figure 1** Tet-regulated induction of Survivin-T34A expression and apoptosis. Melanoma cells stably transfected with tet-Survivin-T34A (T34A-Sur) or tet-wild-type Survivin (WT-Sur) were incubated for the indicated times either in the presence or absence of tet. In this system, transcription is induced in the absence of tet. (a) Lysates (25  $\mu$ g) were subjected to Western blotting with antibodies for Survivin (that recognize endogenous and both mutant and wild-type tet-regulated Survivin) or  $\beta$ -actin. Expression of the respective transgenes can be appreciated relative to the expression of endogenous Survivin seen in cells cultured with tet. (b) T34A-Sur (left panel) and WT-Sur (right panel) cells were cultured in the presence (filled circles) or absence (open circles) of tet as indicated. Cells were fixed and permeabilized, and apoptotic cell fractions were determined by propidium iodide staining and flow cytometry. The data shown are representative of four experiments performed. (c) Annexin V staining of T34A-Sur (open circles) and WT-Sur (filled circles) cells cultured in the absence of tet as indicated. Cells were stained with Annexin V-FITC, and positive cells quantitated by flow cytometry. Error bars reflect s.e.m. from two experiments

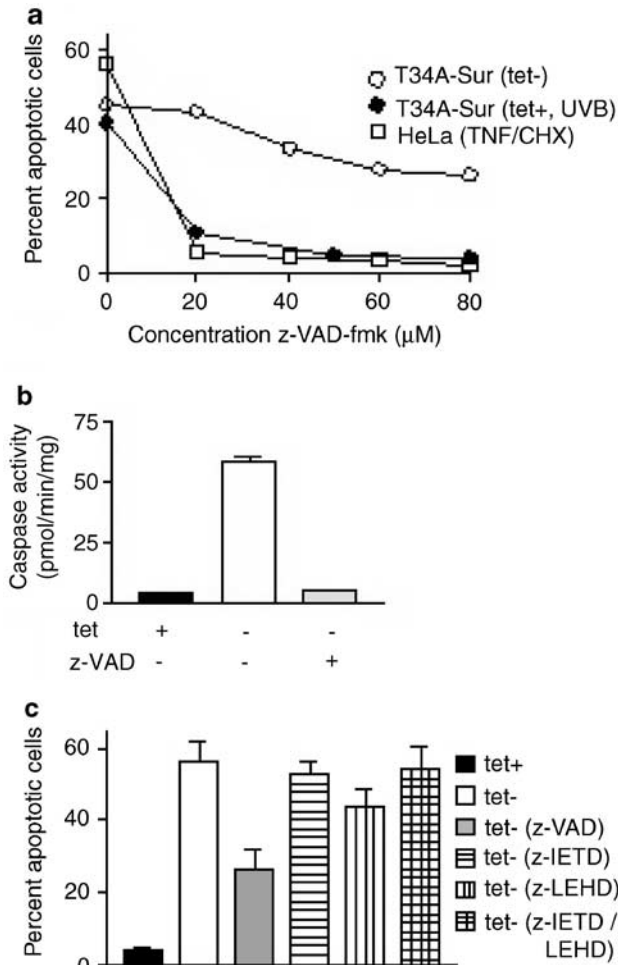
was associated with activation of caspase-9 that was clearly evident by 48 h as indicated by appearance of the 37 kDa cleavage fragment, which was barely visible at 24 h (Figure 2a). Given the inability of Survivin to protect against death receptor signaling (Grossman *et al.*, 2001a), we were somewhat surprised to observe caspase-8 activation in Survivin-targeted cells, as indicated by decreased level of the 55 kDa precursor and appearance of cleavage fragments of 35 kDa (Figure 2b)



**Figure 2** Caspase activation in Survivin-targeted cells. (a) Lysates (100  $\mu$ g) from T34A-Sur cells cultured in the absence of tet for the indicated times were blotted for caspase-3 and -9. Markers indicate caspase-3 precursor (32 kDa) and cleavage fragments (20, 17 kDa), and caspase-9 precursor (45 kDa) and cleavage fragment (37 kDa). (b) Lysates (100  $\mu$ g) from HeLa cells cultured in the absence or presence of TNF- $\alpha$  and cycloheximide, and from T34A-Sur cells cultured in the absence of tet for the indicated times were blotted for caspase-8 and Bid. Markers indicate caspase-8 precursor (55 kDa) and cleavage fragment (35 kDa), and Bid 23 kDa precursor. Additional caspase-8 cleavage fragment (20 kDa) is not shown. Cleaved fragment (tBid) is unstable and not visualized

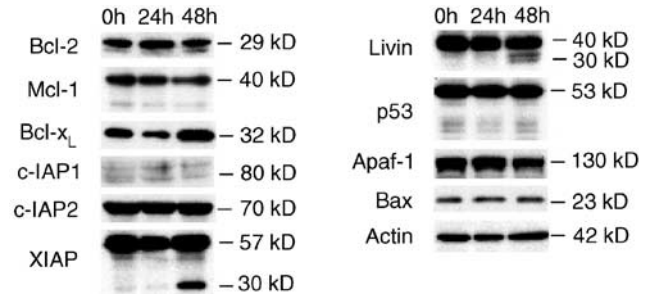
and 20 kDa (not shown) at 48 h. The specificity of these fragments was confirmed by examination of lysates of HeLa cells treated with tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Figure 2b), a potent inducer of caspase-8 activation (Strasser *et al.*, 2000). We then considered the possibility that caspase-8 activation could be responsible for activating caspase-9 through cleavage of Bid (Green, 1998), although this seemed unlikely given that activation of caspase-9 appeared to precede that of caspase-8 (Figure 2a,b). Indeed, Survivin targeting was not associated with detectable Bid cleavage as indicated by persistence even at 48 h of the 23 kDa precursor, which was completely lost in TNF-treated HeLa cells (Figure 2b). Thus, Survivin targeting results in multiple caspase activation, with activation of caspase-9 possibly preceding that of caspase-8 and -3. Caspase activation, however, appears to be a relatively late event in Survivin-targeted cells, occurring well after the onset of Survivin-T34A expression and positive Annexin V staining.

The relatively delayed activation of caspases seen in tet-deprived T34A-Sur cells suggested that caspase activation may not be a critical aspect of apoptosis induced by Survivin targeting. Consistent with this notion, we found that the pan-caspase inhibitor z-Val-Ala-Asp(OMe)-fluoromethyl ketone (z-VAD-fmk) at 20  $\mu$ M could not protect tet-deprived T34A-Sur cells from apoptosis, and was only partially protective at higher concentrations (Figure 3a). By contrast, classically caspase-dependent apoptotic responses



**Figure 3** Caspase-independent apoptosis in Survivin-targeted cells. (a) T34A-Sur cells were incubated in the absence (open circles) or presence of tet after exposure to  $1200 \text{ J/cm}^2$  UVB (filled circles). HeLa cells were treated with TNF- $\alpha$  and cycloheximide (CHX) (open squares). Cells were cultured in increasing concentrations of z-VAD-fmk as indicated, and after 48 h cells were collected and percent apoptotic cells was determined as in Figure 1. The data shown are representative of two experiments performed. (b) T34A-Sur cells were incubated in the presence or absence of tet and  $20 \mu\text{M}$  z-VAD-fmk as indicated, and 48 h later, cell lysates were analysed for caspase activity. Error bars reflect s.e.m. from three measurements. (c) T34A-Sur cells were incubated in the presence or absence of tet, and with  $100 \mu\text{M}$  pan-caspase inhibitor z-VAD-fmk or inhibitors of caspase-8 (z-IETD) or caspase-9 (z-LEHD) as indicated. After 48 h, cells were collected and percent apoptotic cells was determined as in Figure 1. Error bars reflect s.e.m. from two experiments

(Strasser *et al.*, 2000) to UVB and TNF- $\alpha$  were easily blocked by  $20 \mu\text{M}$  z-VAD-fmk in T34A-Sur and HeLa cells, respectively (Figure 3a). A concentration of  $20 \mu\text{M}$  zVAD-fmk was sufficient to neutralize caspase-3 activity (for a fluorogenic caspase substrate) in lysates from tet-deprived T34A-Sur cells (Figure 3b). Selective inhibitors of caspase-8 or -9 were less protective than z-VAD-fmk for Survivin targeting in T34A-Sur cells (Figure 3c). This limited protection by caspase inhibitors suggests that Survivin inhibition also triggers a caspase-independent apoptotic pathway.



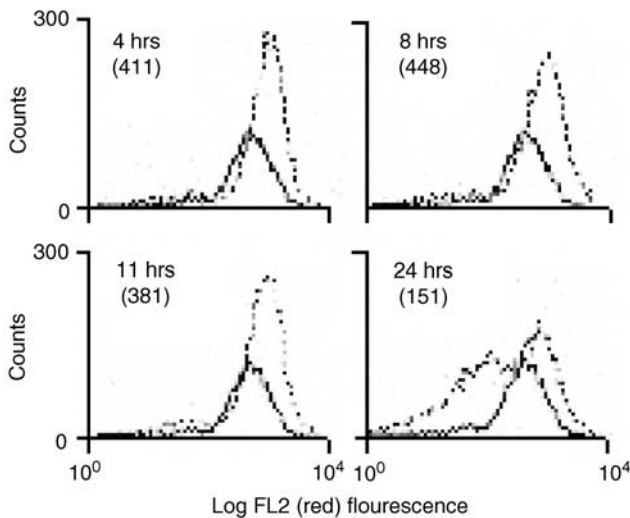
**Figure 4** Survivin targeting does not significantly affect the levels of other apoptotic regulators. Lysates ( $25 \mu\text{g}$ ) were prepared from T34A-Sur cells cultured in the absence of tet for the indicated times. Western blotting was performed for Bcl-2, Mcl-1, Bcl-X<sub>L</sub>, cIAP-1, cIAP-2, XIAP, Livin, p53, Apaf-1, Bax, and Actin as shown. Markers indicate size of proteins and cleavage fragments

### Survivin targeting induces cleavage but does not alter levels of other IAPs

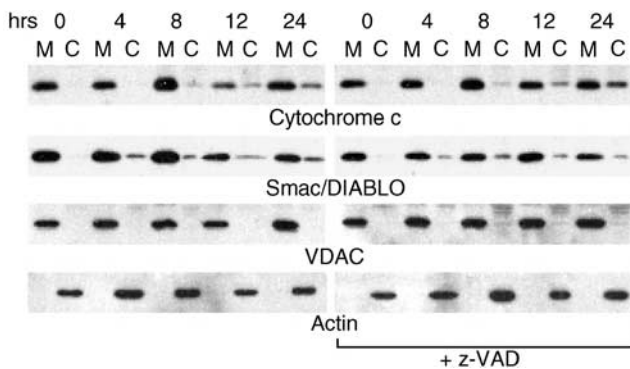
We were curious to examine the levels and possible cleavage of other IAPs in Survivin-targeted T34A-Sur cells, given the previously reported apoptotic cleavage of XIAP (Deveraux *et al.*, 1999; Bowen *et al.*, 2003) and Livin (Bowen *et al.*, 2003), and modulation of cIAP levels in XIAP-knockout mice (Harlin *et al.*, 2001). We did not find alterations in protein levels of cIAP-1, cIAP-2, XIAP, or Livin in T34A-Sur cells following tet withdrawal (Figure 4). Thus, there did not appear to be any compensatory adjustment in IAP expression associated with loss of Survivin function. We did observe modest cleavage of XIAP and Livin at 48 h (Figure 4), corresponding to the time of peak multiple caspase activation. We also examined expression of other apoptotic regulators including antiapoptotic Bcl-2 family members Bcl-2, Mcl-1, and Bcl-X<sub>L</sub>, proapoptotic Bax, as well as proapoptotic regulators p53 and Apaf-1. While there did not appear to be any effect on Bcl-2, Mcl-1, Bcl-X<sub>L</sub>, Bax, p53, or Apaf-1 at 24 h, levels of Mcl-1 and Apaf-1 were decreased by approximately 50% at 48 h (Figure 4). The relatively delayed onset (48 h) of XIAP/Livin cleavage and Mcl-1 decrease, like that of caspase activation, suggested that other events initiated by Survivin targeting may directly mediate apoptosis in T34A-Sur cells.

### Mitochondrial events precede caspase activation in Survivin-targeted cells

Our initial expectation was that mitochondrial events (depolarization, cytochrome *c*, and Smac/DIABLO release) were unlikely to be directly involved in apoptosis induced by Survivin targeting, given previous demonstrations of Survivin acting as an inhibitor of caspases (Conway *et al.*, 2000; O'Connor *et al.*, 2000; Shin *et al.*, 2001) that are downstream of mitochondria in apoptotic pathways (Reed, 2000). However, we saw loss of mitochondrial membrane potential, indicated by a left shift in JC-1 fluorescence and decreased mean fluorescence, which was evident by 11–24 h in tet-deprived T34A-Sur cells (Figure 5).



**Figure 5** Survivin targeting causes mitochondrial depolarization. T34A-Sur cells were cultured over a 24-h period in the presence or absence of tet, and stained with JC-1. Histograms of tet-deprived cells (dotted line) in each panel are overlaid onto the control histogram (cells cultured in the presence of tet, solid line). Shift to the left indicates decreased red fluorescence and loss in mitochondrial transmembrane potential. The geometric mean fluorescence values of tet-deprived cells are indicated in parentheses



**Figure 6** Mitochondrial content release in Survivin-targeted cells. T34A-Sur cells were cultured in the absence of tet, and the presence or absence of  $80 \mu\text{M}$  z-VAD-fmk, for the indicated times. Cells were then fractionated into mitochondrial (M) and cytosolic (C) components, electrophoresed and blotted for cytochrome *c* or Smac/DIABLO as indicated. Blots for VDAC and  $\beta$ -actin confirm equivalent loading among mitochondrial and cytosolic lysates, respectively. Amount loaded per lane of mitochondrial and cytosolic lysates was 20 and  $50 \mu\text{g}$ , respectively

To further characterize the mitochondrial events involved in Survivin targeting, we examined mitochondrial release of cytochrome *c* and Smac/DIABLO in tet-deprived T34A-Sur cells. Cells over a 48-h period were fractionated into mitochondrial and cytosolic components, which were then subjected to Western blotting. Cytochrome *c* and Smac/DIABLO were released from mitochondria into cytosol of T34A-Sur cells and could be detected by 8 and 4 h, respectively, after tet withdrawal (Figure 6). The integrity of mitochondrial and cytosolic fractions was confirmed by staining for voltage-dependent anion channel (VDAC) and  $\beta$ -actin,

respectively (Figure 6). The kinetics of mitochondrial content release was not affected by the presence of z-VAD-fmk (Figure 6), suggesting that these mitochondrial events were not secondary to caspase activation.

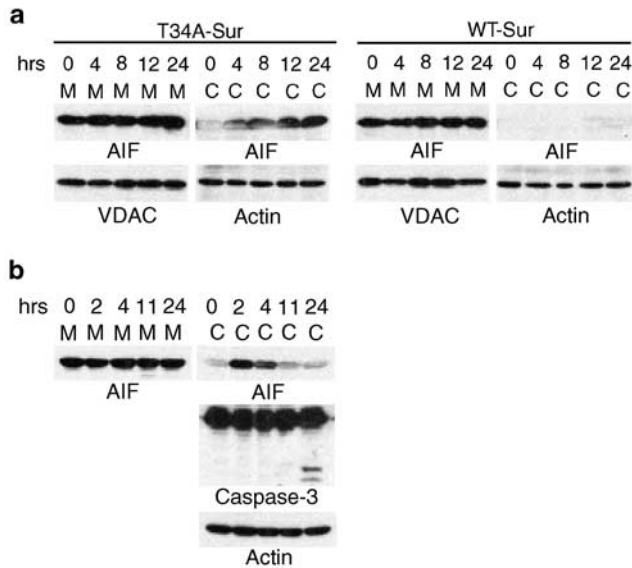
#### Nuclear translocation of mitochondrial AIF

The association of Survivin targeting with a brisk mitochondrial response both preceding, and independent of, caspase activation suggested to us a potential role for AIF given its capacity to mediate DNA fragmentation and mitochondrial cytochrome *c* release in a caspase-independent fashion (Susin *et al.*, 1999). Indeed, we were able to detect movement of AIF from the mitochondrial to the cytosolic compartment of T34A-Sur cells 4 h after tet withdrawal (Figure 7a). There was no AIF translocation observed in tet-deprived WT-Sur cells (Figure 7a). The translocation was detectable even earlier in synchronized (S phase) tet-deprived T34A-Sur cells, with intense cytosolic AIF reactivity seen at 2 h (Figure 7b). Caspase-3 activation was similarly accelerated (from 48 to 24 h), but still occurring well after AIF release (Figure 7b). Fluorescence microscopy and staining for AIF confirmed its mitochondrial location (red/green overlay) in control tet-cultured T34A-Sur cells, and a time-dependent increase in nuclear translocation (red/blue overlay) in tet-deprived T34A-Sur cells (Figure 8). We were able to observe partial nuclear localization of AIF in isolated T34A-Sur cells as early as 4 h after tet withdrawal, and by 24 h many cells exhibited AIF exclusively in the nucleus (Figure 8).

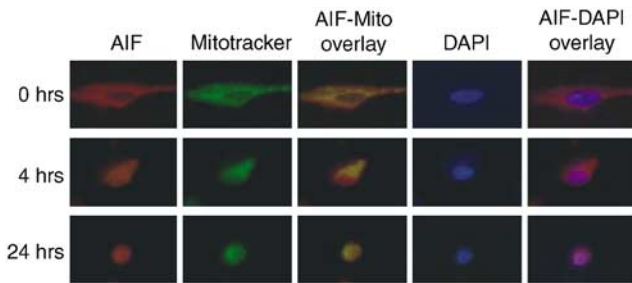
#### Discussion

It has been demonstrated previously that Survivin targeting either by antisense or dominant-negative mutants in melanoma (Grossman *et al.*, 1999a, 2001b) and other malignant cells (Grossman *et al.*, 1999b; Li *et al.*, 1999; Olie *et al.*, 2000; Mesri *et al.*, 2001; Shankar *et al.*, 2001) is sufficient to cause apoptosis. The present challenge has been to elucidate the mechanism of Survivin protection, which will be critical to understanding its function in cancer cells and developing potential therapeutic applications. Using an inducible Survivin dominant-negative mutant, we define here the nature and kinetics of the apoptotic events following Survivin inhibition in melanoma cells. We found that the earliest proapoptotic event in Survivin-targeted cells was nuclear translocation of mitochondrial AIF, known to trigger both apoptotic mitochondrial events and caspase-independent DNA fragmentation.

Survivin has generally been thought to exert an antiapoptotic function through caspase inhibition, a view that originated from structure–function considerations and quickly gained support from various experimental systems. At about the time that other IAPs were shown to function as caspase inhibitors (Deveraux *et al.*, 1997; Roy *et al.*, 1997), Survivin was identified and recognized to contain an IAP-defining BIR domain



**Figure 7** Mitochondrial release of AIF in Survivin-targeted cells. **(a)** T34A-Sur and WT-Sur cells were cultured in the absence of tet for the indicated times. Mitochondrial (M, 20  $\mu$ g) and cytosolic (C, 50  $\mu$ g) lysates were electrophoresed and blotted for AIF. Blots for VDAC and  $\beta$ -actin confirm equivalent loading among mitochondrial and cytosolic lysates, respectively. **(b)** T34A-Sur cells were cultured for 24 h in the presence of 2 mM thymidine, washed, then cultured in the absence of tet for the indicated times. Mitochondrial (M, 20  $\mu$ g) and cytosolic (C, 50  $\mu$ g) lysates were electrophoresed and blotted for AIF, caspase-3, and actin as indicated



**Figure 8** Survivin targeting triggers rapid nuclear translocation of AIF. T34A-Sur cells were cultured in the absence of tet for 0, 4, or 24 h as indicated. Cells were stained with anti-AIF (red), Mitotracker (green), and DAPI (blue), and images were overlaid as shown. Yellow color indicates colocalization of AIF and mitochondria. Purple color indicates colocalization of AIF and nucleus

(Ambrosini *et al.*, 1997). Subsequent studies demonstrated activation of both caspase-9 (O'Connor *et al.*, 2000; Mesri *et al.*, 2001) and caspase-3 (Li *et al.*, 1998; Mesri *et al.*, 2001) in Survivin-targeted cells, and Survivin binding to caspase-9 (O'Connor *et al.*, 2000), caspase-3 (Tamm *et al.*, 1998; Conway *et al.*, 2000; Shin *et al.*, 2001), and caspase-7 (Tamm *et al.*, 1998; Shin *et al.*, 2001), but not caspase-8 (Tamm *et al.*, 1998). This observed spectrum of anticaspase activity, encompassing both initiator (caspase-9) and effector (caspase-3 and caspase-7) caspases, was consistent with functional studies (Li *et al.*, 1998; Grossman *et al.*, 2001a), localizing the regulatory function of Survivin to the

mitochondrial apoptotic pathway. However, examination of Survivin crystal structures (Chantalat *et al.*, 2000; Verdecia *et al.*, 2000) did not reveal a putative caspase-binding ('hook and sinker') region as seen in other IAPs (Altieri, 2003), and subsequent efforts to demonstrate Survivin binding to caspase-3 have not been successful (Banks *et al.*, 2000; Verdecia *et al.*, 2000). In addition, two groups (Mesri *et al.*, 2001; Shankar *et al.*, 2001) have shown that Survivin targeting is associated with mitochondrial content release, which generally occurs upstream of caspase-9 and -3 activation in apoptotic pathways (Reed, 2000). Taken together, these recent data have cast some doubt as to whether caspase inhibition is indeed the primary antiapoptotic function of Survivin.

These previous studies (Li *et al.*, 1998; O'Connor *et al.*, 2000; Mesri *et al.*, 2001; Shankar *et al.*, 2001) were not designed to resolve whether caspase activation was a primary or secondary phenomenon occurring in Survivin-targeted cells. The novel approach we have taken here, using an inducible targeting system, has allowed us to assess caspase activation and other apoptotic events in a relative chronological context. We initially envisioned two possible scenarios by which blocking Survivin could result in caspase activation. First, expression of Survivin-T34A could interfere with constitutive caspase binding by endogenous Survivin, resulting in liberation or activation of caspases. Second, Survivin targeting could disrupt complexes of endogenous Survivin with Smac/DIABLO (Du *et al.*, 2000), with displaced Smac/DIABLO resulting in increased sequestration of other IAPs and thereby enhancing caspase activation. In either case, our expectation was that expression of the dominant-negative Survivin mutant would result in an abrupt increase in caspase activity. The observed delay (24–48 h) in caspase activation, and activation of multiple caspases including caspase-8 (which has never been shown to interact with Survivin), suggested that caspase activation was likely a secondary apoptotic event occurring in Survivin-targeted cells. We also considered the possibility that perturbation of Survivin function could modulate expression of other IAPs, as was observed with cIAP-1 and cIAP-2 in XIAP-deficient mice (Harlin *et al.*, 2001), and thereby affect caspase activity. However, there did not appear to be any effect on expression of IAPs associated with loss of Survivin function. We did observe partial cleavage of XIAP and Livin and decreased levels of Mcl-1, but like the kinetics of caspase activation, the relatively delayed onset suggested that other events triggered by Survivin targeting were more likely to be initiating apoptosis in T34A-Sur cells.

We were struck by the rapidity of mitochondrial events in Survivin-targeted cells. Release of Smac/DIABLO and cytochrome *c*, and mitochondrial depolarization, were detected almost immediately following induced expression of Survivin-T34A. Although there are reports of caspase feedback to mitochondria (Ricci *et al.*, 2003) and caspase-mediated release of cytochrome *c* (Bossy-Wetzels and Green, 1999) and AIF (Zamzami *et al.*, 2000), our finding that mitochondrial events

preceded caspase activation is more consistent with a scenario whereby caspases are activated secondary to mitochondrial depolarization and content release. Inability of the pan-caspase inhibitor z-VAD-fmk to affect mitochondrial content release in our experimental system supports this notion. The earliest event observed in Survivin-targeted cells was nuclear translocation of mitochondrial AIF, which appeared to occur simultaneously to the expression of mutant Survivin in T34A-Sur cells. Translocation of AIF, known to induce mitochondrial cytochrome *c* release and subsequent caspase activation as well as caspase-independent nuclear fragmentation (Susin *et al.*, 1999; Daugas *et al.*, 2000), is the most likely explanation for mitochondrial events preceding caspase activation and caspase-independent apoptosis seen in Survivin-targeted cells. Although others have described release of cytochrome *c* (O'Connor *et al.*, 2000; Mesri *et al.*, 2001) and AIF (Shankar *et al.*, 2001) following Survivin inhibition, the present study is the first, to our knowledge, to establish a precise chronological context for these mitochondrial events. We suggest that the primary antiapoptotic function of Survivin appears to be antagonism of the AIF pathway and protection from caspase-independent apoptosis, with caspase activation occurring secondary to mitochondrial events that are also induced by AIF (Figure 9). Studies by Altieri and co-workers (Li *et al.*, 1998; O'Connor *et al.*, 2000) have suggested that cells are most susceptible to Survivin targeting in mitosis. We found that detection of apoptotic events, namely AIF translocation and caspase-3 activation, was accelerated in synchronized cells but the sequence of these events was not altered, as AIF translocation still preceded caspase activation. We have also observed AIF translo-

cation and caspase-independent apoptosis in various Survivin-expressing melanoma lines treated with other apoptotic stimuli (TL and DG, unpublished observations), suggesting the presence of a common apoptotic pathway in melanoma cells. Future genetic targeting experiments will be required to determine if AIF is required and sufficient for apoptosis induced by Survivin targeting.

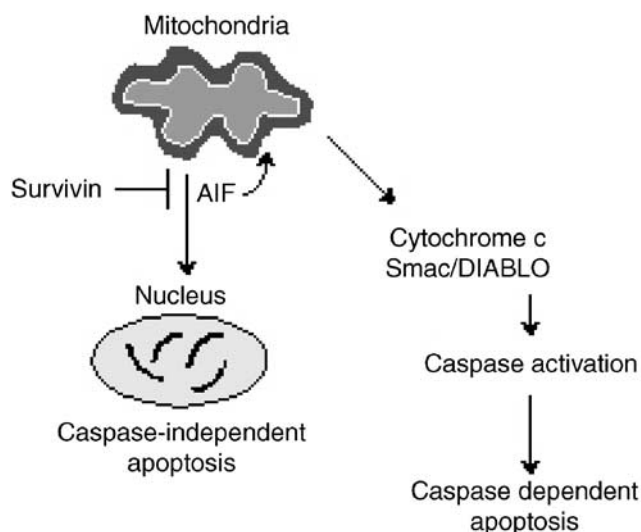
AIF is highly conserved from mammals to worms (Wang *et al.*, 2002) and is critical for some apoptotic responses (Joza *et al.*, 2001); yet, its molecular mechanism of action and physiologic role in adult organisms are poorly understood. Crystallization studies (Mate *et al.*, 2002; Ye *et al.*, 2002) have revealed two distinct domains, one involved in direct DNA binding and another with oxidoreductase enzymatic activity, although this second domain appears not to be required for proapoptotic activity (Miramar *et al.*, 2001). Nuclear translocation of AIF is preceded by the activation of nuclear poly(ADP-ribose) polymerase-1 (PARP-1) (Yu *et al.*, 2002), but it is unclear whether any of the resulting ADP-ribosylated nuclear proteins or the accompanying NAD<sup>+</sup> depletion directly mediate the translocation event. Recent immunohistological studies have revealed that Survivin resides in nuclear as well as cytosolic compartments (Fortugno *et al.*, 2002), so it is possible that Survivin could modulate AIF by direct interactions with a nuclear factor such as PARP-1. Interestingly, a Survivin isoform lacking exon 3 (Survivin-Ex3) has been described that has a mitochondrial localization signal and accumulates in the nucleus when overexpressed (Mahotka *et al.*, 1999).

An antagonistic relationship between Survivin and AIF provides further justification for the broad expression of Survivin in cancers (Velculescu *et al.*, 1999). It is intriguing to speculate that AIF translocation and caspase-independent apoptosis may represent a backup apoptotic mechanism in normal cells should caspases fail or become dysregulated. Indeed, various caspases have been found mutated or blocked in normal and malignant cells (Reed, 1999, 2000). Tumor expression of Survivin may confer suppression of AIF activity and eliminate this backup mechanism, thus further enhancing apoptotic resistance. Understanding the integration of Survivin and AIF in the cellular apoptotic machinery will have important implications for future development of apoptosis-based therapies, designed either to promote apoptosis in cancer or prevent it in degenerative diseases.

## Materials and methods

### Cells

The generation of subclones of the human melanoma cell line YUSAC2 (Grossman *et al.*, 1999a) stably transfected with tet-regulated wide-type human *Survivin* (WT-Sur, clone 4C7) and *Survivin* Thr<sup>34</sup> → Ala (T34A-Sur, clone F5C4) cDNA has been described previously (Grossman *et al.*, 2001b). In this 'tet-off' system (Shockett *et al.*, 1995), transcription is induced by removal of tet from the culture medium, thus these cells were



**Figure 9** Diagram depicting antagonism of Survivin and AIF pathways. Survivin restrains AIF in mitochondria. Targeting Survivin causes release of AIF, which translocates to the nucleus and mediates caspase-independent apoptosis. AIF also triggers the conventional mitochondrial apoptotic pathway, characterized by mitochondrial release of cytochrome *c* and Smac/DIABLO that lead to caspase activation

maintained in the presence of tet as described (Grossman *et al.*, 2001b). HeLa cells (ATCC) were maintained in DMEM containing 10% FCS and antibiotics.

### Reagents

Rabbit polyclonal antibody reactive with human Survivin and Survivin-T34A has been described (Grossman *et al.*, 2001b), and is commercially available from NOVUS Biologicals (Littleton, CO, USA). Rabbit polyclonal antibodies against precursor and cleavage fragments of caspase-3 (sc-7148), caspase-8 (sc-7890), and caspase-9 (sc-7885) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against Bcl-2 (sc-492), Mcl-1 (sc-819), Bcl-X<sub>L</sub> (sc-7195), Bax (sc-493), cIAP-1 (sc-7943), cIAP-2 (sc-7944), and cytochrome *c* (sc-7159) were from Santa Cruz. Rabbit anti-Smac/DIABLO was obtained from Imgenex (San Diego, CA, USA). Goat polyclonal antibodies against Bid (sc-6538) and AIF (sc-9416) were also from Santa Cruz. Rabbit antibody to VDAC was obtained from Affinity BioReagents, Inc. (Golden, CO, USA). Mouse monoclonal anti-p53 (Ab-6) was obtained from Calbiochem (San Diego, CA, USA). Mouse monoclonal antibodies against XIAP (hILP, clone 48) and  $\beta$ -actin (clone AC-74) were obtained from Transduction Laboratories (San Diego, CA, USA) and Sigma Chemical Co. (St Louis, MO, USA), respectively. Rabbit antiserum generated against Apaf-1 (clone 846) and Livin were kindly provided by Dr Yuri Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA) and Dr Gary Kasof (AstraZeneca, Worcester, MA, USA), respectively. Caspase inhibitors z-VAD-fmk (pan-caspase), z-IETD-fmk (caspase-8), and z-LEHD-fmk (caspase-9) from Enzyme Systems Products (Livermore, CA, USA) were solubilized in DMSO and stored at  $-20^{\circ}\text{C}$ . TNF- $\alpha$  and cycloheximide were both from Sigma and stored at  $-20^{\circ}\text{C}$ . Thymidine was also from Sigma and freshly prepared prior to use.

### Western blotting

Cell lysates were prepared, electrophoresed, transferred to PVDF membranes, and blocked with nonfat milk as described previously (Grossman *et al.*, 1999a). Blots were reacted with primary antibodies to Survivin (0.5  $\mu\text{g}/\text{ml}$ ), caspase-3 (1:400), caspase-8 (1:200), caspase-9 (1:200), Bid (1:200), Bcl-2 (0.2  $\mu\text{g}/\text{ml}$ ), Mcl-1 (0.2  $\mu\text{g}/\text{ml}$ ), Bcl-X<sub>L</sub> (0.5  $\mu\text{g}/\text{ml}$ ), cIAP-1 (0.5  $\mu\text{g}/\text{ml}$ ), cIAP-2 (0.2  $\mu\text{g}/\text{ml}$ ), XIAP (0.25  $\mu\text{g}/\text{ml}$ ), Livin (1:3000), p53 (0.1  $\mu\text{g}/\text{ml}$ ), Apaf-1 (1:3000), Bax (0.3  $\mu\text{g}/\text{ml}$ ), cytochrome *c* (1:400), Smac/DIABLO (1  $\mu\text{g}/\text{ml}$ ), VDAC (1:750),  $\beta$ -actin (1:30,000), or AIF (1:250) in PBS containing 5% nonfat milk and 0.1% Tween-20 for 1–2 h at room temperature. After washing, blots were stained with appropriate peroxidase-conjugated secondary antibody and bands were visualized using enhanced chemiluminescence as described (Grossman *et al.*, 1999a).

### Apoptosis induction

Tet-regulated apoptosis was induced in T34A cells by washing adherent cells twice in HBSS and then culturing in medium (Grossman *et al.*, 2001b) without tet. In some experiments, cells were first synchronized in S phase by the addition of 2 mM thymidine to the culture medium for 24 h. For UVB-induced apoptosis, T34A cells were washed in PBS, exposed to 1200 J/m<sup>2</sup> UVB (Grossman *et al.*, 2001a), and harvested after 24 h. HeLa cells were treated for 24 h with a combination of 10 ng/ml TNF- $\alpha$  and 10  $\mu\text{g}/\text{ml}$  cycloheximide.

### Apoptosis detection

Late-stage apoptosis was assessed by total cellular DNA content using propidium iodide and flow cytometry as described previously (Grossman *et al.*, 1999a). Early-stage apoptosis was assessed by phosphatidylserine staining using an Annexin V kit (Santa Cruz) according to the manufacturer's instructions. Briefly, cells were washed twice with cold PBS, resuspended in binding buffer ( $1 \times 10^5$  cells in 0.1 ml), and 2.5  $\mu\text{l}$  of FITC-conjugated Annexin V was added. After incubation at room temperature for 15 min in the dark, an additional 400  $\mu\text{l}$  of binding buffer was added, and the cells were analysed within 1 h by flow cytometry. Mitochondrial depolarization was assessed by JC-1 fluorescence, following the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). Briefly,  $2 \times 10^5$  cells were resuspended in 1 ml culture medium containing 2.5  $\mu\text{g}/\text{ml}$  JC-1 dye, and incubated for 10 min in the dark at  $37^{\circ}\text{C}$  with intermittent agitation. Cells were then pelleted, washed twice, and then resuspended in 0.3 ml PBS for flow cytometry. Two band filters (525 and 590 nm) were used to monitor dye fluorescence, with green–orange electronic signal compensation set at 4% and orange–green electronic signal compensation at 10%.

### Cellular fractionation

Cells ( $1 \times 10^7$ ) were washed in cold PBS, and then resuspended in 1 ml cold homogenization buffer (pH 7.4) containing 0.3 M mannitol (Sigma), 10 mM potassium hydroxide, 10 mM HEPES (Calbiochem, La Jolla, CA, USA), 0.1% BSA (Sigma), 0.2 mM EDTA, and  $1 \times$  protease inhibitor cocktail (Complete, Roche Applied Science, Indianapolis, IN, USA). A homogenate was prepared with a 2 ml glass Dounce Tissue homogenizer (VWR Scientific Products, New Haven, CT, USA) on ice, using a type-A pestle 10 times and a type-B pestle 10 times, and then transferred to a 1.5 ml microfuge tube. After centrifugation ( $\times 1000 g$ ) for 10 min to remove cellular debris and nuclei, the supernatant was collected and spun ( $\times 14000 g$ ) at  $4^{\circ}\text{C}$  for 15 min. The resulting supernatant (cytosolic fraction) was collected, and the pellet was then washed twice and resuspended in homogenization buffer (mitochondrial fraction). Fractions were diluted in SDS-containing sample buffer, aliquotted and stored at  $-20^{\circ}\text{C}$ .

### Caspase-3 activity

Cell lysates (45  $\mu\text{l}$ ) prepared by sonication were combined with 5  $\mu\text{l}$  (500  $\mu\text{M}$  final concentration) of fluorogenic caspase-3 substrate Ac-DEVD-AMC (Peptides International Inc., Louisville, KY, USA) in triplicate in a microtiter plate according to the manufacturer's instructions. The plate was agitated for 5 min, incubated at  $37^{\circ}\text{C}$  for 2 h, then fluorescence readings were taken at excitation and emission wavelengths of 360 and 460 nm, respectively. Caspase-3 activity was proportional to the AMC fluorescence, and normalized to lysate protein content to be expressed as pmol/min/mg protein.

### Fluorescence microscopy

Cells were grown on 18 mm coverslips (Fisher scientific, Houston, TX, USA) and incubated for 15 min at  $37^{\circ}\text{C}$  in a medium containing 100 nM MitoTracker CMXRos (Molecular Probes) to stain mitochondria. Cells were fixed in PBS containing 4% paraformaldehyde for 15 min at room temperature, washed with PBS, then permeabilized in PBS containing 0.2% Triton X-100 for 5 min. For AIF staining,

cells were washed and then incubated with 1:50 dilution of anti-AIF antibody in PBS for 1 h at 37°C. After washing, cells were stained with 1:500 dilution of FITC-conjugated donkey anti-goat-IgG (Santa Cruz) in PBS for 1 h at 37°C. Then the coverslips were inverted and adhered to the glass slides using a mounting solution containing 4,6-diamidino-2-phenylindole (DAPI, Sigma) as described previously (Grossman *et al.*, 1999a). Images from fluorescence patterns of the cells were obtained using an Axiophot microscope (Zeiss, New York, NY, USA), and processed as one-color images or two-color overlays, and Adobe Photoshop software (Mountain View, CA, USA). Colors were assigned as follows to optimize overlays: AIF (red), Mitotracker (green), and DAPI (blue).

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