IF_1 limits the apoptotic-signalling cascade by preventing mitochondrial remodelling

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Mitochondrial structure has a central role both in energy conversion and in the regulation of cell death. We have previously shown that IF_1 protects cells from necrotic cell death and supports cristae structure by promoting the oligomerisation of the F_1F_0-ATP synthase. As IF_1 is upregulated in a large proportion of human cancers, we have here explored its contribution to the progression of apoptosis and report that an increased expression of IF_1, relative to the F_1F_0-ATP synthase, protects cells from apoptotic death. We show that IF_1 expression serves as a checkpoint for the release of Cytochrome c (Cyt c) and hence the completion of the apoptotic program. We show that the progression of apoptosis engages an amplification pathway mediated by: (i) Cyt c-dependent release of ER Ca^{2+}, (ii) Ca^{2+}-dependent recruitment of the GTPase Dynamin-related protein 1 (Drp1), (iii) Bax insertion into the outer mitochondrial membrane and (iv) further release of Cyt c. This pathway is accelerated by suppression of IF_1 and delayed by its overexpression. IF_1 overexpression is associated with the preservation of mitochondrial morphology and ultrastructure, consistent with a central role for IF_1 as a determinant of the inner membrane architecture and with the role of mitochondrial ultrastructure in the regulation of Cyt c release. These data suggest that IF_1 is an antiapoptotic and potentially tumorigenic factor and may be a valuable predictor of responsiveness to chemotherapy.

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The efficiency of the intrinsic pathway of apoptosis depends on the release of mitochondrial pro-apoptotic proteins (e.g., Cytochrome c (Cyt c)) into the cytosol, where they trigger the caspase-dependent execution of the cell. Other mitochondria-associated events are: (a) changes in electron transport, (b) loss of transmembrane potential (ΔΨ_m)^4, (c) Ca^{2+} overload^6 and (d) re-organization of mitochondrial ultrastructure. The regulatory pathways that govern the dynamics of mitochondrial fusion/fission events are the object of general interest for their critical role in both cell physiology and pathology. Their relevance in apoptosis is also widely acknowledged. Alterations in ultrastructure occur in numerous diseases and are also a feature of apoptosis. The narrow tubular junctions that connect mitochondrial cristae to the inner boundary membrane support core functions such as: (i) creating gradients of small molecules (adenosine diphosphate), (ii) ions’ homeostasis (e.g. Ca^{2+})^11 and (iii) segregation of the respiratory chain complexes. Cyt c localizes in the intermembrane space, and disruption of tubular junctions is required to support its release into the cytosol at the onset of apoptotic cell death.

The endogenous inhibitor of the ATPase, IF_1, is a small, basic, heat-stable protein composed of 80–84 amino acids (∼10 kDa) in mammals and predominantly compartmentalized inside the mitochondrial matrix. IF_1 has the unique capacity to inhibit, through a non-competitive mechanism, the adenosine triphosphate (ATP)-hydrolysing activity of the F_1F_0-ATP synthase without affecting the synthesis of ATP during oxidative phosphorylation. The protein is α-helical along most of its length (~90 Å) and at low pH (<6.7) becomes active as a dimer, making this the only condition in which its inhibitory role is exerted. The dimerization of IF_1—which is critical for the inhibition of the F_1F_0-ATP synthase—involves the C-terminal regions of two monomers (residues 37–84), which form an antiparallel double-stranded coiled-coil unit stabilized by complementary hydrophobic interactions between the two helices. By assessing the role of IF_1 on resting mitochondrial physiology and morphology, we recently reported a prominent effect on cristae number and alignment, suggesting that IF_1 has a role in the regulation of mitochondrial ultrastructure.

Mitochondrial ultrastructure is governed by: (a) the mitofilin/Fcj1 complex, which is essential for cristae development; (b) the pro-fusion protein Optic atrophy 1 (Opa1) and (c) the dimerization of the F_1F_0-ATP synthase. The latter is promoted by IF_1, with increased density of cristae, contributing to the morphology of single mitochondria and that of the whole network. In addition, dimerization of the...
IF₁Fₐ-ATP synthase was recently shown to protect cells from starvation,²⁴ highlighting the importance of molecules with a role in this. Beside the outcome on mitochondrial morphology, IF₁ is key to the preservation of cellular ATP during hypoxia or ischemia,¹⁹ but its outcome on pathways mediating apoptosis remains controversial. IF₁ expression is greatly increased in a number of human cancers,²⁵,²⁶ and recently it has been proposed to interact with the canonical pro-survival pathway, NF-κB,²⁷ priming an adaptive response. Even though this was explained via an IF₁-mediated inhibition of mitochondrial oxidative phosphorylation and a resultant increased production of ROS—an aspect which is still controversial²⁸—this implies a mitochondrial downstream effect that would impinge on signaling pathways of cancer cells.

The shaping of the outer mitochondrial membrane is sustained by the pro-fission GTPase Dynamin-related protein 1 (Drp1)²⁹,³⁰ and, during apoptosis, by the activity of the pro-apoptotic member of the Bcl-2 family Bax,³⁰,³¹ whose oligomerization facilitates mitochondrial outer membrane permeabilization.³² Drp1 translocation to mitochondria is a Ca²⁺-dependent process, as its activity is regulated by phosphorylation and dephosphorylation at several amino acid residues: a rise in [Ca²⁺] induces the Ca²⁺/Calmodulin-dependent activation of Calmodulin kinase I alpha, which phosphorylates Drp1 at Ser600, and Calcineurin, which dephosphorylates Drp1 at Ser637. Both events are crucial to induce the translocation of Drp1 onto the outer mitochondrial membrane.³³,³⁴ Ca²⁺ per se contributes to apoptosis by facilitating mitochondrial fission when the organelles are overloaded with the ion.³⁵

Moreover, Snyder and co-workers suggested that Cyt c, once released from mitochondria, binds to inositol 1,4,5-triphosphate receptors (IP₃Rs), promoting the release of Ca²⁺ from the endoplasmic reticulum (ER) and increasing Ca²⁺ accumulation into mitochondria.³⁶,³⁷ This implies that proteins that limit disassembly of the mitochondrial network, cristae remodeling and release of Cyt c could also affect Ca²⁺ signaling in apoptosis.

Our data demonstrate that IF₁ may modulate apoptosis by regulating mitochondrial morphology, so limiting Cyt c release. They support a model whereby Cyt c promotes ER Ca²⁺ release, which leads to activation of Drp1 and recruitment of Bax to the outer mitochondrial membrane, where it permeabilises the membrane inducing further Cyt c release; this self-sustaining positive feedback amplification pathway may account for the all-or-none release of mitochondrial Cyt c.³⁸ IF₁—via modulation of the mitochondrial architecture—represents an early checkpoint in this pathway, limiting the efficient progression of the apoptotic cascade and protecting therefore the cells from the apoptotic cell death.

Results

IF₁ counteracts apoptotic remodeling of the mitochondrial structure. The IF₁:FₐFₒ-ATP synthase expression ratio was manipulated in HeLa cells as in our previous studies,¹⁹,²³ using a blank vector as control. Cells expressing different levels of IF₁ were exposed to the pro-apoptotic agent Staurosporine (STS), a broad-spectrum protein kinase inhibitor³⁹ and, after 4 h of treatment, prepared for transmission electron microscopy analysis. The same experiment was repeated using etoposide.

As shown in Figure 1Aa, an extensive remodeling of cristae architecture following treatment with both stimuli characterized IF₁ knock down cells (−/−IF₁), while the shape of cristae remained intact in cells overexpressing IF₁ (+/IF₁). The number of cristae per mitochondrion was quantified and plotted in Figure 1Ab.

Using the recombinant mitochondrially targeted green fluorescent protein, we then analyzed the mitochondrial network morphology (Figure 1Ba). In control cells, mitochondria were fragmented and disrupted after STS treatment. In −IF₁ cells the network was completely disrupted after only 4 h. However, within the same timeframe, no significant alteration was seen in +IF₁ cells (Figure 1Bb).

IF₁ reduces apoptotic redistribution of Cyt c. Subsequently, we investigated the entity of Cyt c release during apoptosis in control and −/−IF₁ cells via confocal imaging analysis. For this purpose, we transfected cells with the recombinant Cyt c-GFP chimera³³ and quantified Cyt c redistribution by measuring the ratio between its mean fluorescence values and the relative standard deviation of the fluorescence signal (see Materials and Methods; Figure 2Aa).

After only 2 h of STS treatment, 11% of control cells showed Cyt c redistribution, while in +IF₁ cells this was limited to just 3%, and suppression of Cyt c release remained even after 6 h of treatment. Conversely, in −IF₁ cells, the release of Cyt c was greater than control (Figure 2Aa). Values of each condition between 0 and 3 h of treatment were quantified and shown in Figure 2Ab. After 7–8 h of STS treatment, Cyt c release from +IF₁ cells increased, and there was no longer any significant difference.

ΔΨₘ was measured simultaneously during Cyt c-GFP redistribution (Supplementary Figure S1a). In tetramethylrhodamine, methyl ester-loaded control and +IF₁ cells, this was done at intervals of 30 min over 8 h of STS treatment. ΔΨₘ values, measured at every time point, were normalized between the initial baseline and the fully depolarized mitochondrial state, which was obtained by adding FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) at the end of each experiment.

We observed that Cyt c release occurred in tandem with mitochondrial depolarization and that the events took place simultaneously and interdependently in both the control and +IF₁ groups (values reported in Figures 2Ba and Bb).

Cyt c release and the loss of membrane potential were both significantly suppressed in +IF₁ cells during STS treatment (Figure 2B).

Although it remains contentious,³⁷ it has been suggested that the formation of the mitochondrial permeability transition pore (mPTP) may promote the loss of ΔΨₘ and, possibly, Cyt c release during apoptosis,⁴⁰ the diverse incidence of the two events in the two groups of cells could be, therefore, due to differences in mPTP opening.³⁶ Hence, we repeated the analysis in the presence of Cyclosporine A, a pharmacological
**Figure 1** Mitochondrial ultrastructure and remodeling during apoptosis depends on IF1 levels. (A) Transmission electron microscope micrographs of HeLa cells before and after treatment with 1 μM STS and 100 μM etoposide (ETO). (a) An increased number of cristae was evident in +IF1 cells, as highlighted by the quantification in panel (b) \((n = 3, *P < 0.05, **P < 0.01)\). (B) Representative confocal images of HeLa cells transfected with mtGFP before and after challenging with 1 μM STS; an overall preservation of the mitochondrial network was seen in cells overexpressing IF1. Quantification of mitochondrial volume in all conditions is reported in panel (b), indicating that IF1 ratio of expression is involved in the morphological adaptation of mitochondria to apoptosis \((n = 3, **P < 0.01)\)
inhibitor of mPTP. In control cells, Cyclosporine A significantly suppressed both redistribution of Cyt c (Figure 2Ba) and dissipation of $\Delta \Psi_m$ (Figure 2Bb) after only 5–6 h of STS treatment, while in + IF1 cells the activity of Cyclosporine A was delayed, and a same effect was reached after 7–8 h. These data support a role for the mPTP in the loss of $\Delta \Psi_m$ during apoptosis.

**Figure 2** IF1 limits Cyt c release and apoptotic cell death. (A) Prototypical images of Cyt c release from mitochondria in control, IF1 overexpressing or IF1 knockdown HeLa cells after challenging with (a) 1 μM STS. The redistribution of Cyt c from the mitochondrial compartment into the cytosol was visualized cotransfecting cells with GFP-tagged Cyt c. The graph in panel (b) shows the percentages of control, IF1 overexpressing and IF1 knockdown HeLa cells releasing Cyt c from mitochondria during treatment with 1 μM STS. Data were acquired every hour over a period of 8 h (n = 4 cover slips, *P < 0.05, **P < 0.01). (B) Bar charts showing the percentage of control and + IF1 HeLa cells with (a) redistributed Cyt c-FITC and (b) dissipated $\Delta \Psi_m$ at various time points of STS treatment in the absence or presence of the mPTP inhibitor Cyclosporine A (CsA). Notably, in + IF1 cells a CsA-mediated inhibition of Cyt c-FITC release was only evident at 7–8 h, while in control cells CsA had clear effects at 5–6 h, the time point required by the drug to mediate a protective effect on $\Delta \Psi_m$ in both conditions.

A apoptotic cell death is modulated by the IF1:F1$_{ATP}$ synthase ratio. Along with the retention of Cyt c and $\Delta \Psi_m$ in the initial phases of apoptosis, the exposure of phosphatidylserine on the plasma membrane and the activation of Caspase 3 were also limited by IF1 overexpression and facilitated by its downregulation (Figures 3Aa and Ab).
**A**

- Control
- +IF1
- -IF1

**B**

- Control
- +IF1
- -IF1

**C**

- STS
- IF1

**D**

- YFP
- α-Bax
- TO-PRO
- Merge

**Graphs and Figures:**

- **Graph A:** Bar graphs showing Caspase Activation and Annexin-V Positive Cells.
- **Graph B:** Bar graphs showing % Living Cells.
- **Graph C:** Western blots showing Whole cell lysates and Mito fractions.
- **Graph D:** Fluorescence micrographs showing YFP, α-Bax, TO-PRO, and Merge.
Cell survival was measured following 12 h exposure to Staurosporine, etoposide or C2-ceramide. A significant protection from apoptosis was observed in + IF1 cells and the opposite in those downregulated for the gene (Figure 3B). Perhaps surprisingly, increased IF1:F1:F0-ATPsynthase ratio of expression was also protective from cell death triggered by the agonistic anti-CD95 antibody (α-CD95), which activates the extrinsic pathway of apoptosis (Supplementary Figure S1bii).42 Although not recapitulated by the deletion of the gene, as −IF1 cells undergo cell death in equal measure of control, we asked whether IF1 altered the expression of Caspase 8, which links the extrinsic and intrinsic pathways of apoptosis (Supplementary Figure S1b). This analysis revealed that Caspase 8 expression was not modulated by changes in IF1 expression but that in +IF1 cells treatment with α-CD95 promotes a more prominent cleavage in the p43/41 fragments, which would account for an increased activation of the pathway.

**IF1 overexpression limits the activity of Bax and Drp1 on mitochondria.** During apoptosis, Bax translocates onto the MOM and; after a conformational shift with exposure of the C-terminus, inserts into it, facilitating the release of Cyt c.43 We therefore assessed Bax expression levels in control and + / −IF1 cells. Bax appeared to be evenly expressed in all the cell types at resting physiology (Figure 3Ca).

Fractions of purified mitochondria isolated after 4 h of treatment with STS were probed with an anti-Bax antibody; although almost no staining was detectable in +IF1 cells, the observed band intensity in −IF1 cells was much greater than control cells (n = 5) (Figure 3Cc). In addition, this trend was also confirmed by immunocytochemical analysis (Figure 3D).

Mitochondrial outer membrane permeabilisation, effected by Bax, depends on the remodeling of membranes, for which the recruitment of Drp1 to mitochondria is required.30 Mitochondrial fractions tested for Drp1 expression showed that downregulation of IF1 facilitates the relocation of Drp1 onto mitochondria during apoptosis, as the protein was retrieved in greater amount when compared with the cytosolic fraction, whereas the opposite was seen following overexpression of IF1 (Figure 4Aa). As for the analysis of Bax, we used an immunocytochemical analysis to confirm the data (Figure 4Ac).

In addition, Drp1 activation differed between mitochondria expressing diverse levels of IF1, as extracts of −IF1 cells were more strongly stained with the anti-pospho-Drp1(S616) antibody that recognizes the active form of the protein (Figure 4B).

These data suggest that IF1 overexpression reduces Drp1 translocation to mitochondria, as well as the interdependent translocation and oligomerisation of Bax. We therefore expressed the dominant-negative mutant of Drp1 (Drp1K38A) in + / −IF1 cells, and measured the percentage of living cells after 12 h incubation with STS. Under these conditions, we saw no differences between the three conditions compared with cells expressing naive levels of Drp1 (Figure 4C). The same outcome was obtained in Drp1−/− cells (data not shown).

**IF1 limits Ca2+ mobilization during apoptosis activation.** According to Boehning et al.,36 Cyt c binds to IF1:F1s on the adjacent ER at the beginning of apoptosis, thus enhancing Ca2+ release, mitochondrial Ca2+ loading and opening of the mPTP, so amplifying the release of Cyt c. As the redistribution of Cyt c and the loss of mitochondrial potential were significantly delayed in +IF1 cells, we explored the potential role of Ca2+ signaling in this pathway. Cells transfected with IF1 cDNA and control cells were loaded with Fura-2, AM, treated with STS and imaged over 8 h. Alternative patterns of [Ca2+]i signals were seen with varying frequencies in control and +IF1 cells (Figures 5Aa and Ab). The majority of +IF1 cells (85.60%) showed no change in [Ca2+]i compared with ~40% of control cells, in which a significant percentage showed either a progressive increase (13.09%) or spikes (46.86%) in [Ca2+]i, which were very rarely observed in +IF1 cells. IF1 overexpression reduced STS-induced changes in [Ca2+]i, and this effect correlated with the delay in Cyt c release from mitochondria (as quantified in Figure 2Ab).

The differences in [Ca2+]i patterns may be attributed to differences in the initial release of Cyt c, which may modulate ER Ca2+ signaling so amplifying Cyt c release to completion. We therefore measured the ER Ca2+ content using Thapsigargin (Tg, 500 nM) at several time points following exposure to STS. Representative traces of changes in [Ca2+]i following STS treatment and Tg application are shown in Figures 5Ba and Bb. As reported in Supplementary Figure S1ciii, [Ca2+]iER was substantially depleted in control cells compared with +IF1 cells, consistently with the increased release of ER Ca2+ seen in control cells.

Remarkably, as a chance observation, we noted that STS altered the capacitative Ca2+ influx in both control and +IF1 cells; in fact, while in untreated cells [Ca2+]i remained elevated after addition of Tg (Figure 5Ba), it decreased to

![Figure 3](image)

**Figure 3** IF1:F1:F0-ATP synthase ratio of expression regulates cell response to apoptosis modulating the activity of Bax. (A) Bar charts displaying the percentage of control and IF1 up/downregulated HeLa cells with (a) caspase activation and (b) positive Annexin-V staining after 14 h treatment with 1 μM STS. Remarkably, in +IF1 cells caspase activation and exposure of phosphatidylserine to the extracellular environment were lower than in control cells, while an opposite situation was seen in −IF1 cells (P < 0.05, **P < 0.01). (B) Chart reporting the percentage of living cells after 12 h treatment with 1 μM STS, 20 μM C2-Car or 100 μM etoposide (ETO) in control, +IF1 and −IF1 HeLa cells. An augmented resistance to all apoptotic stimuli was evident in +IF1 cells, whereas −IF1 cells showed an opposite effect (n = 3, **P < 0.05, ***P < 0.01). (C) (a and b) Western blot analysis of cells modulated for IF1 expression, showing that total cellular levels of Bax were not affected by the expression levels of IF1. Band densities have been quantified and normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The western blot in panel (c) shows instead that, during STS-induced apoptosis (4 h treatment with 1 μM STS), Bax accumulation was limited in cells overexpressing IF1. After treatment, cells devoid of IF1, exhibited higher levels of Bax in the mitochondrial fraction, indicating an increased mitochondrial recruitment during apoptosis (band densities, quantified and normalized to the I-chain of F1:F1-ATP synthase, are reported in panel (d); n = 6; P < 0.05, **P < 0.01). (D) Representative immunocytochemistry images showing Bax translocation to mitochondria in control, +IF1 and −IF1 HeLa cells after 4 h treatment with 1 μM STS.
basal levels in STS-treated cells (Figure 5Bb). A similar pattern occurred up to 6 h of STS treatment, as reported in Supplementary Figures S1ci and cii.

Uptake of Ca\textsuperscript{2+} by mitochondria was then measured using Rhod-2, AM (Figure 5Ca). After 2 h of STS treatment, mitochondria of + IF\textsubscript{1} cells showed no significant increase in
Ca\textsuperscript{2+} content, while this was elevated in control cells. Quantified data are plotted in Figure 5Cb.

In cells overexpressing IF\textsubscript{1}, the limited mobilization of Ca\textsuperscript{2+} also reduced the activity of the serine/threonine protein phosphatase Calcineurin. Activity of Calcineurin, the Ca\textsuperscript{2+}-dependent activator of Drp1, was reduced after treatment with STS in + IF\textsubscript{1} cells, with an opposite result in − IF\textsubscript{1} cells (Figure 5D).

The hierarchy of the events we attribute to IF\textsubscript{1} is presented as a cartoon in Figure 5E.

**Discussion**

Mitochondrial fragmentation and cristae remodeling augment Cyt\textsubscript{c} release to completion within minutes during apoptosis.\textsuperscript{2,38} Proteins involved in the regulation of mitochondrial dynamics have an active part in this process, opposing or facilitating the structural changes of the organelle.\textsuperscript{49} For example, Opa1 maintains the shape of cristae, reduces the mobilization of Cyt\textsubscript{c} and delays cell death,\textsuperscript{45} while Drp1, which is essential for mitochondrial fission, has an opposite effect, by sustaining the oligomerization of Bax.\textsuperscript{30} Here, we demonstrate that IF\textsubscript{1} also contributes to the structural re-arrangement of mitochondria during apoptosis, modifying the mobilization of Cyt\textsubscript{c} and so altering the downstream cascade of events.

Our data suggest that IF\textsubscript{1} overexpression limits mitochondrial release of Cyt\textsubscript{c}, so that the downstream events that amplify Cyt\textsubscript{c} release and complete the apoptotic program are delayed. Overexpression of IF\textsubscript{1} preserved cristae morphology (Figure 1) and reduced Cyt\textsubscript{c} release (Figure 2), Ca\textsuperscript{2+} signaling (Figure 5), Drp1 activation (Figure 4) and Bax oligomerisation (Figure 3) for 7 h after STS treatment (Figures 1, 2, and 4), also delaying: (i) caspase activation, (ii) phosphatidylserine translocation and (iii) cell death (Figure 3). The opposite was seen in cells in which IF\textsubscript{1} expression was suppressed, as just 2 h of treatment with STS were sufficient to produce an almost complete re-distribution of Cyt\textsubscript{c} (Figure 2Aa).

Interestingly, IF\textsubscript{1} was also protective when apoptosis was activated via the CD95/APO-1/Fas signaling pathway (Supplementary Figure S1bi). Because the Caspase 8 cascade, via cleavage of BID,\textsuperscript{46} merges on the mitochondrial pathway. The increased activation retrievable in +IF\textsubscript{1} cells in which apoptosis is delayed, does imply a prominent mitochondria mediated effect.

Mitochondria were not immune from the apoptotic-associated events when IF\textsubscript{1} was overexpressed, as both migration of Cyt\textsubscript{c} and dissipation of ΔΨ\textsubscript{m} did take place, even if at a later stage—after 7 h of incubation with STS. Even though at this time point the events on which protection by IF\textsubscript{1} is primed in the initial phases are statistically normalized, it has to be noted that (a) the trend toward delay of Cyt\textsubscript{c} release and ΔΨ\textsubscript{m} dissipation remains similar to those statistically proven at 3–4 h and (b) modulation of both events occurring earlier in time are associated with a significant protection from cell death when IF\textsubscript{1} is overexpressed (Figure 3).

Notably, such a regulation of apoptosis efficiency by IF\textsubscript{1} appears independent of the canonical action of the protein as an inhibitor of the reverse mode of the F\textsubscript{1}F\textsubscript{o}-ATP synthase.\textsuperscript{47} One might predict that apoptosis itself should predispose to a similar scenario, with reversal of the ATP synthase and accelerated ATP consumption so that ΔΨ\textsubscript{m} dissipation would occur faster in + IF\textsubscript{1} cells. This was not the case: even though IF\textsubscript{1} dimers were still present after STS treatment with a greater degree (Supplementary Figure S1d), these did not influence ΔΨ\textsubscript{m} that dissipated concomitantly with the release of Cyt\textsubscript{c} and, consequently, with the permeabilisation of mitochondria.

The regulation of apoptosis by IF\textsubscript{1} embeds other elements of the process, such as (a) the multidomain protein Bax and (b) Drp1. Cuezva and co-workers have proposed that IF\textsubscript{1} overexpression influences proteins of the Bcl-2 family by increasing the expression of Bcl-XL.\textsuperscript{27} For this reason, we explored whether expression of Bax was also altered, but we found no changes in its expression despite alternative degrees of IF\textsubscript{1} expression. We only observed changes during active apoptosis and just in the mitochondrial localization of the pro-apoptotic molecule, an event that was almost absent in IF\textsubscript{1}-overexpressing cells (Figures 3Cc and Cd). Even though such an effect may be due to the increment of the anti-apoptotic counterparts of the same family—hence in line with the findings of an increased presence of Bcl-XL—other events may still modify the anchoring capacity of Bax, and the translocation/activation of the pro-fission protein Drp1 is one of these (Figures 4A and B).\textsuperscript{30} The effects of IF\textsubscript{1} on mitochondrial morphology (Figure 1) primed the analysis based on the recently proposed model of Martinou and colleagues, in which the degree of mitochondrial Drp1 is instrumental for promoting Bax-contributed remodeling of the membranes.\textsuperscript{30} This explains our findings whereby in − IF\textsubscript{1} cells a dramatic increase in mitochondrial translocation of Drp1 is followed by activation (Figure 4B).

Thus IF\textsubscript{1}, although intramitochondrially localized, can reach out and affect mediators of apoptosis localized in the cytosol (e.g., Drp1), as confirmed by the fact that modulation of IF\textsubscript{1} expression was ineffective in altering the apoptotic response when cells expressed the dominant-negative mutant of Drp1 (Figure 4D). A similar scenario—with different actors—was also proposed in a previous relevant work, with a role for NF-κB as the underlying proliferative pathway via which IF\textsubscript{1} exploited its anti-apoptotic and tumorigenic role.\textsuperscript{27} As this was linked to concomitant changes in mitochondrial respiration.

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**Figure 4** Drp1 activation and relocation on mitochondria depends on IF\textsubscript{1}. (A) (a and b) Western blot (WB) analysis indicating that Drp1 recruitment to mitochondria during apoptosis (still 4 h treatment with 1 μM STS) was increased after knocking down IF\textsubscript{1}, (band density quantification for mitochondrial and cytosolic Drp1 is reported below; n = 4, *P < 0.05, **P < 0.01). (c) Immunocytochemical analysis of Drp1 recruitment on mitochondria—still at 4 h treatment with STS. In cells devoid of IF\textsubscript{1}, Drp1 colocalized on mitochondria at higher levels, as highlighted in the representative images reported and relative intensity. (B) During apoptosis, (a) activated Drp1 (phosphorylated at Ser616) was detectable in WB analysis in the mitochondrial fraction of − IF\textsubscript{1} cells (with relative band density quantification reported in panel (b); n = 3, *P < 0.05, **P < 0.01). (C) Chart reporting the percentage of living cells after 12 h treatment with 1 μM STS in control, + IF\textsubscript{1} and − IF\textsubscript{1} HeLa cells co-transfected with the enzymatically inactive mutant Drp1K38A cDNA. The Drp1-dependent effect of IF\textsubscript{1} is revealed by the fact that, when Drp1 was not active, all cell populations were protected from apoptosis, with no detectable differences in the percentage of living cells after STS treatment (n = 2, *P < 0.05, **P < 0.01)
that we have not observed under any condition, we remain unsure how to reconcile these different data sets.

It is possible to speculate that reduced translocation of Drp1 to mitochondria depends on alterations in the ratio of hFis1:10 to per mitochondrial—considering the organelle’s elongation after Atpi1 upregulation—however, because at resting physiology we find the same amount of Ca2+ overload, which promotes swelling and opening of the mPTP.31 In addition, Drp1 dephosphorylation by Calcineurin, and its subsequent translocation to mitochondria, is also mediated by Ca2+. As IF1, delayed the release of Cyt c and suppressed ER Ca2+ mobilization in both cytosolic and mitochondrial compartments, we explored the activity of Calcineurin as the logical link between Ca2+ and Drp1.33,52 Consistent with this model, in + IF1 cells activation of the protein phosphatase was much reduced.

Doubts remain about whether the elongated shape of mitochondria affects the contact sites between ER and mitochondria and, ultimately, the Ca2+-buffering capacity of mitochondria; however, the kinetics of cytosolic [Ca2+] during STS treatment being equal in both the control and + IF1 cells after Tg challenge, we ruled out this option.

Thus, we propose that, besides its role in ischemia, IF1 also protects from apoptotic cell death, primarily by influencing mitochondrial ultrastructure and membrane remodeling.19,23

Our data support a model of apoptosis that involves a cascade of actions, which will serve as an amplification mechanism to ensure the rapid, complete and efficient release of all mitochondrial Cyt c within a short time frame. Some elements of the scheme, outlined in Figure 5, have been controversial, but it turns out that increased expression of IF1 proves an invaluable tool to interrupt this cascade, revealing the importance of downstream processes. It is therefore tempting to propose that the increased expression of IF1 described in human cancers may point to a tumorigenic role for IF1, given its antiapoptotic action; assaying IF1 levels in cancer biopsies may thus provide a useful prognostic indicator for chemotherapy responsiveness.

**Materials and Methods**

Experiments were carried out in Human Cervical Adenocarcinoma cells (HeLa), wild-type and Drp1−/− mouse embryonic fibroblasts. Cells were transfected as explained previously.19

**Investigating mitochondrial morphology.** Confocal images of mitochondria were acquired as previously described.23 For electron micrograph analysis, transfected cells were grown on gridded cover slips and 36 h after transfection identified by fluorescence microscopy and their localization marked. Cells were processed as described in precedences19 and images acquired with a 2 JEOL 1010 transmission electron microscope (Jeol Ltd., Tokio, Japan). Acquired images were analyzed using Image J (National Institutes of Health, Bethesda, MD, USA) and ‘Lucida’ (Kinetic Imaging, Andor Technology plc., Belfast, UK) to define the number of cristae per mitochondrion.

**Cell death analysis.** At a confluence of ~50%, cells cultured on 0.22 mm borosilicate cover slips were transfected with IF1, cDNA, IF1 siRNA or yellow fluorescent protein (YFP) cDNA (as a control). After 36 h of transfection, cells were incubated for 12 h at 37 °C, 5% CO2 with one of the following apoptosis inducers: 1 mM STS (Calbiochem 569 397; Merck Millipore, Billerica, MA, USA), 100 μM etoposide (Sigma-Aldrich Co, St. Louis, MO, USA), 20 μM C2-ceramide or 1 μg/ml anti-CD95 (Clone CH11) Ab (No. 05-201, Millipore, Millipore). After that time, a period established to achieve significant death but leaving a reasonable number of surviving cells for adequate data collection, cells were washed with recording medium and cover slips were assembled into a chamber for confocal imaging. Annexin V and poly-caspases analysis were carried out according to the manufacturers’ instructions (BD Pharmingen TM 550 911; BD Biosciences, San Jose, CA, USA), Poly-Caspases FLICA kit, category No. 92, IT) with a Zeiss LSM confocal laser scanning microscope (Carl Zeiss Group, Oberkochen, Germany) (inverted configuration on an Axiovert 200), using a ‘Fluar’ × 40/0.30 oil immersion objective. Images were analyzed using the LSM Image Browser software (Carl Zeiss Group).

**Evaluation of Cyt c redistribution.** Cyt c release was followed using the Cyt c-GFP fusion protein. Cells were seeded on 0.22 mm borosilicate cover slips and, when ~50% confluent, cotransfected with either IF1, and Cyt c-GFP cDNAs (− IF1; cells), IF1 siRNA (hs ATTP1F1 2; S00308812, Qiagen, Hilden, Germany) and Cyt c-GFP cDNA (− IF1; cells) or an empty vector and Cyt c-GFP cDNA (control cells). Thirty-six hours after transfection, each cover slip was mounted into a chamber for live-cell imaging and inserted into the thermostatted chamber of a Zeiss LSM 510 confocal laser scanning microscope (inverted configuration on an Axiovert 200 frame), with temperature constantly set at 37 °C. Images were acquired using a ‘Fluar’ × 40/0.30 oil immersion objective and an Argon laser (488 nm line; 1% laser power) to excite GFP, which fluorescence was captured through the HFT 486 dichroic mirror with BP 500/530 band-pass filter. At the start of each experiment, one image was captured for baseline measurements; then, 1 μM STS was added to recording medium and images were hourly acquired until end (8 h after STS addition for + IF1 cells, 4 h for −IF1 cells). Analysis was

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**Figure 5** IF1 overexpression counteracts Ca2+ mobilization and Calcineurin (CaN) activation during apoptosis. (A) (a) Traces of control and + IF1, HeLa cells loaded with 5 μM Fura-2, AM, treated with 1 μM STS and imaged for 8 h, monitoring changes in cytosolic Ca2+ levels. Three types of cytosolic [Ca2+] patterns were observed. Data summarized in panel (b) illustrate that a significantly higher percentage of +IF1 cells showed no changes in cytosolic Ca2+ levels as compared with control cells. Conversely, a higher percentage of control cells showed rises and spike-like increases in cytosolic [Ca2+] during STS treatment. (n = 3 cover slips, 15–29 cells per cover slip, *P < 0.05). (B) Representative traces of changes in [Ca2+] in control and + IF1, HeLa cells exposed to 1 μM STS and then challenged with 500 nM Tg to specifically inhibit the activity of sarco/ER Ca2+-ATPases. The release of Ca2+ in the cytosol was measured to infer ER Ca2+ content. In the absence of a) STS, ER Ca2+ content was not significantly different in control and + IF1 cells; (b) after 2 h of STS treatment, [Ca2+] in control, + IF1 cells was instead greatly depleted in control cells, but better maintained in + IF1 cells. (C) Representative (a) images and (b) kinetics of mitochondrial Ca2+ handling monitored with Rhod-2, AM, during treatment with 1 μM STS in control and + IF1, HeLa cells. Although control cells exhibited a rise in [Ca2+] after challenging with STS, in + IF1 cells mitochondrial Ca2+ levels were stably maintained (normalized signals: 1 h, Control: 1.34 ± 0.39; + IF1: 2.23 ± 0.49, 2 h, Control: 2.21 ± 0.07; + IF1: 1.28 ± 0.13; 3 h, Control: 1.18 ± 0.05; + IF1: 0.92 ± 0.08; n = 4, 10 cells per condition, *P < 0.05). (D) Histogram reporting the enzymatic activity of Cyt c in control, + IF1 and − IF1 cells. Cells were treated for 4 h with STS to induce apoptosis or with DMSO as a control. The activity of Cyt c has been measured and reported as nmol of P0 released (values are normalized to control). Notably, in − IF1 cells this value was higher at resting conditions and after triggering apoptosis, indicating a higher activation of CaN, while IF1 overexpression inhibits this event (*P < 0.05; **P < 0.01). (E) Model for IF1, functional role in apoptosis. IF1 preserves the inner mitochondrial membrane structure by increasing the cristae stability,1 reducing the release of Cyt c and hence avoiding the Cyt c-induced mobilization of ER-stored Ca2+. This limits CaN activity45 and hence Drp1 recruitment/activation on mitochondria, also hindering the function of Bax.5 The delayed massive mitochondrial release of Cyt c prevents the formation of the apoptosome into the cytochrome and the execution of apoptosis
carried out using AQM Advance 6 software (Andor, Belfast, UK) to obtain ratios of the S.D. of the green-fluorescent signal relative to the mean (S.D./Mean) for the Cyt c-GFP fluorescence emission; this gives a quantifiable measure of Cyt c redistribution in each region of interest analyzed. In brief, when the Cyt c-GFP chimeric protein is localized into mitochondria, the S.D. is high because there are bright pixels (corresponding to mitochondria) interspersed with black pixels (corresponding to cytoplasm). Once Cyt c-GFP redistributes through the cytosol, the S.D. falls as the signal is evenly distributed throughout the cell; this needs to be normalized with respect to the mean signal simply because S.D. is also a function of the overall intensity. The S.D./Mean values of Cyt c-GFP signal in all the analyzed cells were binned to explore the distribution. Changes in distribution of the ratio following treatment with STS from time 0–4 h were plotted for control and + IF1 cells. The distribution for control cells displayed a leftward shift from time 0–4 h after STS treatment compared with that of + IF1 cells, showing that more control than + IF1 cells had undergone Cyt c redistribution. Control cells analyzed at 4 h from STS addition were expected to consist of two populations, one showing Cyt c release and the other one without. Indeed, the distribution plot for control cells at 4 h was fitted well by the sum of two Gaussian distributions representing the two sub-populations of cells. An intersection point between the two Gaussian populations was demarcated and threshold S.D./Mean values of the Cyt c-GFP signal were set to define the two sub-populations. Cells with S.D./Mean ratios of <0.6 were defined as those that showed Cyt c redistribution, and cells with S.D./Mean >0.7 were considered as those that did not show Cyt c release from mitochondria.
While supernatants were centrifuged at 100,000 g, incubating mitochondrial pellets with 138 mM NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, and cells were washed twice with modified Hanks’ Buffered Salt Solution (HBSS 138 mM NaCl, 5.4 mM KCl, 0.25 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1.0 mM MgSO₄, 4.2 mM NaHCO₃, pH 7.4). Each cover slip of cells was assembled into a purpose-built chamber, a fresh aliquot of modified HBSS with or without 1 μM STS was added to the cells and placed on the stage of a Leica TCS S5P II confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Temperature inside the environmental chamber of the microscope was constantly maintained at 37°C. Images were acquired for 8 h using a HCX PL APO 40 ×/1.25-0.75 oil immersion CS objective and collecting Fura-2 emission at 505 nm in the ratio mode with excitation of the dye at 340 and 380 nm light (using an UV laser, a 400 long pass dichroic mirror and a 510/540 band pass filter). Settings were adjusted to minimize phototoxicity. Acquired images were analyzed with the LSM Image Browser software.

Western blotting. At a confluence of ~50%, cells cultured on 100 mm Petri dishes were transfected with either IF1-YFP cDNA or YFP cDNA (as a control). After 36 h of transfection, cells were incubated with 1 μM STS and treated with or without 1 μM STS for the required number of hours were challenged with Tg, a sarcoplasmic reticulum Ca²⁺-ATPase inhibitor, to prevent Ca²⁺ reuptake into the ER.

For experiments measuring ER Ca²⁺ concentration, cells loaded with Fura-2, AM and treated with 1 μM STS for the required number of hours were challenged with Tg, a sarcoplasmic reticulum Ca²⁺-ATPase inhibitor, to prevent Ca²⁺ reuptake into the ER. ER Ca²⁺ levels were analyzed with the red-fluorescent Ca²⁺ indicator Rhod-2, AM. Cells were seeded on 22 mm borosilicate cover slips and transfected with either IF1-YFP cDNA or YFP cDNA when ~50% confluent. After 36 h, cells were loaded with 5 μM Rhod-2 in the presence of 0.05% (v/v) pluronic acid in modified HBSS (45 min incubation at 37°C, 5% CO₂). After four washes with modified HBSS to remove excess dye, cover slips were assembled into a live-cell imaging chamber and an aliquot of modified HBSS with 1 μM STS (or DMSO (dimethyl sulfoxide) vehicle as a control) was added. Cover slips were then seeded into the thermostatted chamber of a Zeiss LSM 510 Confocal Laser Scanning Microscope (inverted configuration on an Axiovert 200 frame) and cells were imaged using a ‘Fluar’ ×10/0.3 oil immersion objective and a green Helium-Neon laser (543 nm line; 1% laser power) with a BP 565/615 band-pass filter. Settings were chosen to reduce photobleaching of the fluorophore and the pinhole set to give an optical slice of ~2 μm. Acquisitions were taken every 30 min to obtain a time course of mitochondrial Ca²⁺ -images. Images were analyzed with the LSM Image Browser.

Calcineurin activity assay. At a confluence of ~50%, cells cultured on 100 mm Petri dishes were transfected with IF1, IF1-YFP cDNA, or an empty vector (as a control). After 36 h of transfection, cells were incubated with 1 μM STS supplemented medium (4 h incubation at 37°C, 5% CO₂) to induce apoptosis; DMSO vehicle was used as a control. After treatment, Calcineurin activity was measured using a Calcineurin cellular activity assay kit (Enzo Life Sciences Inc., Farmingdale, NY, USA), following manufacturer’s instructions.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions
MC and MRD designed the study and wrote the paper. DF, CHT, MC and AS performed the experiments.

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