Mortalin-mediated and ERK-controlled targeting of HIF-1α to mitochondria confers resistance to apoptosis under hypoxia

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ABSTRACT

Hypoxia inducible factor-1 (HIF-1) is the main transcriptional activator of the cellular response to hypoxia and an important target of anticancer therapy. Phosphorylation by ERK1 and/or ERK2 (MAPK3 and MAPK1, respectively; hereafter ERK) stimulates the transcriptional activity of HIF-1α by inhibiting its CRM1 (XPO1)-dependent nuclear export. Here, we demonstrate that phosphorylation by ERK also regulates the association of HIF-1α with a so-far-unknown interaction partner identified as mortalin (also known as GRP75 and HSPA9), which mediates non-genomic involvement of HIF-1α in apoptosis. Mortalin binds specifically to HIF-1α that lacks modification by ERK, and the HIF-1α–mortalin complex is localized outside the nucleus. Under hypoxia, mortalin mediates targeting of unmodified HIF-1α to the outer mitochondrial membrane, as well as association with VDAC1 and hexokinase II, which promotes production of a C-terminally truncated active form of VDAC1, denoted VDAC1-ΔC, and protection from apoptosis when ERK is inactivated. Under normoxia, transcriptionally inactive forms of unmodified HIF-1α or its C-terminal domain alone are also targeted to mitochondria; stimulate production of VDAC1-ΔC and increase resistance to etoposide- or doxorubicin-induced apoptosis. These findings reveal an ERK-controlled, unconventional and anti-apoptotic function of HIF-1α that might serve as an early protective mechanism upon oxygen limitation and promote cancer cell resistance to chemotherapy.

KEY WORDS: Hypoxia, Apoptosis, HIF-1, Mortalin, Mitochondria, ERK

INTRODUCTION

Exposure to low oxygen conditions (hypoxia) triggers changes in gene expression mediated by the hypoxia-inducible factors (HIFs) (Semenza, 2012). Gene activation by HIFs results in metabolic reprogramming, stimulates angiogenesis and impacts proliferation of cells subjected to hypoxia. Adaptation of cancer cells to the hypoxic microenvironment of solid tumors, as well as survival of normal cells in ischemic tissues, therefore, depends on the activation of HIFs (Keith et al., 2012). HIFs are composed of oxygen-regulated HIFα subunits and HIFβ (known as ARNT). Expression of HIF-1α, the most studied and widely expressed HIF isoform, is continuous but, in the presence of a physiological oxygen concentration, the protein fails to accumulate as it is degraded by the proteasome following its hydroxylation by oxygen-dependent prolyl hydroxylases (PHDs) and its polyubiquitylation by a pVHL-containing E3 ligase complex (Schofield and Ratcliffe, 2005). When oxygen levels drop, HIF-1α very rapidly accumulates as a result of inhibition of hydroxylation and subsequent stabilization. HIF-1α is then transported into the nucleus, forms a heterodimer with ARNT, binds to hypoxia-response DNA elements (HREs) and promotes expression of hypoxia target genes.

In addition to oxygen-dependent stabilization of HIF-1α, HIF-1α is also controlled by mechanisms not directly involving oxygen but implicating oncogenic signaling pathways, protein interactions and diverse modifications (Dengler et al., 2014). Direct phosphorylation by kinases such as GSK3, PLK3, CDK1 and ATM (Kietzmann et al., 2016) affect HIF-1α protein stability, whereas others, such as ERK1 and ERK2 (ERK1/2, also known as MAPK3 and MAPK1, respectively; hereafter referred to as ERK) (Mylonis et al., 2008) and CK18 (CSNK1D) (Kalouzi et al., 2010), control its activity by affecting nuclear accumulation or heterodimerization with ARNT, respectively. More specifically, phosphorylation of HIF-1α at Ser641 and/or Ser643 by ERK masks an adjacent CRM1 (XPO1)-dependent nuclear export signal (NES) and inhibits HIF-1α nuclear export, thereby increasing its nuclear concentration and transactivation ability (Mylonis et al., 2008).

Adaptation to hypoxia involves metabolic reprogramming driven by upregulation of the HIF-1-target genes that stimulate uptake of glucose, glycolysis, lactate production and secretion, glycogen storage and glutamine catabolism (Brahimi-Horn et al., 2011), and promote accumulation of triglycerides in lipid droplets (Kourt et al., 2015; Mylonis et al., 2012) as well as, in contrast, repressing mitochondrial metabolism and oxidative phosphorylation (Semenza, 2011). To ensure cell survival under hypoxia, HIF-1 also stimulates mitochondrial autophagy and upregulates the expression of anti-apoptotic genes of the Bcl-2 family (Sendel and Hengartner, 2014), thereby also increasing resistance of cancer cells to chemotherapy (Wilson and Hay, 2011).

Hypoxia also affects mitochondrial-mediated apoptosis by interfering with the function of outer mitochondrial membrane (OMM) proteins. Hypoxia stimulates the expression of hexokinase II (HK-II, also known as HK2), a hexokinase isoform that is upregulated in various tumors and is associated with higher tumor grade and mortality (Roberts and Miyamoto, 2015). HK-II binds to the OMM through its interaction with the voltage-dependent anion channel 1 (VDAC1), the major porin of OMM. Mitochondrial HK-II not only contributes to the glycolytic phenotype of cancer cells by coupling mitochondrial production of ATP to glucose phosphorylation (the first step of glycolysis) but also prevents apoptosis by inhibiting binding of pro-apoptotic factors to the OMM and blocking release of cytochrome c, the intrinsic trigger of caspase activation.

VDAC1 itself is also involved in apoptotic signaling by interacting with Bcl-2 family proteins and by affecting OMM
permeabilization and escape of apoptogenic proteins (Shoshan-Barmatz et al., 2015). In several different cancer cell lines, hypoxia triggers proteolytic processing of VDAC1 to produce a C-terminally truncated active form called VDAC1-ΔC (Brahimi-Horn et al., 2012, 2015). Production of VDAC1-ΔC depends on HIF-1 and HK-II and is associated with enlarged mitochondria and increased resistance to drug-induced apoptosis. Finally, VDAC1 also interacts with mortalin, also known as GRP75 and HSPA9 (Schwarzer et al., 2002; Szabadkai et al., 2006). Mortalin resides mainly on the OMM and its overexpression is associated with chemotherapy resistance, and cancer progression and metastasis (Lee, 2014).

The involvement of HIF-1 in regulation of apoptosis has so far been assumed to be indirect through transcriptional activation of genes encoding pro- or anti-apoptotic factors. We demonstrate in this work an unconventional, non-genomic role of HIF-1α as an ERK-regulated effector of apoptosis through its association with mortalin, VDAC1 and HK-II on the mitochondrial surface.

RESULTS
ERK-controlled interaction of HIF-1α with mortalin
To identify phosphorylation-dependent interactions of HIF-1α, GST-tagged forms of its ERK-targeted domain (ETD, amino acids 616-658) carrying either phospho-deficient (ETD-SA, Ser641Ala and Ser643Ala) or phosphomimetic (ETD-SE, Ser641Glu) mutations were used as baits in pulldown assays with total HeLa cell protein extracts. A single 75-kDa protein could be detected associated specifically with ETD-SA (Fig. 1A) and was identified by mass spectrometric analysis (Table S1) as mortalin. Immunoblotting confirmed the identity of mortalin (Fig. 1A), and immunoprecipitation of endogenous HIF-1α demonstrated its interaction with mortalin under hypoxia (Fig. 1B). This interaction was enhanced when ERK activation was blocked by selective MEK inhibitor U0126, which substantially reduced the overall phosphorylation of HIF-1α at serine residues (Fig. S1A), thus confirming that mortalin binds preferably to HIF-1α lacking ERK-mediated modifications (hence referred to as unmodified HIF-1α). In the reverse experiment, immunoprecipitation of mortalin confirmed its association with unmodified HIF-1α (Fig. S1B) and, in addition, showed its inability to associate with HIF-2α (Fig. S1C), as suggested by the uniqueness of the ETD amino acid sequence in HIF-1α.

Treatment with U0126 also caused translocation of HIF-1α to outside of the nucleus (Fig. 1C), strong inhibition of its transcriptional activity (Fig. 1E) and reduction in the expression of the HIF-1-target genes VEGFA, BNIP3 and NDRG1 (Fig. 1F). Furthermore, non-nuclear HIF-1α largely colocalized with mortalin (Fig. 1C). The physical interaction between HIF-1α and mortalin and its stimulation by U0126 were also confirmed with an in situ proximity ligation assay (in situ PLA; Fig. 1D). HIF-1α–mortalin complexes could be already detected under hypoxia in the absence of U0126 and their number significantly increased when HIF-1α phosphorylation by ERK was inhibited.

ERK-controlled and mortalin-mediated association of HIF-1α with the mitochondrial surface
The physical association of HIF-1α with mortalin suggested that unmodified HIF-1α might also associate with mitochondria. Indeed, hypoxia-induced endogenous HIF-1α lost its predominant nuclear location and colocalized to substantial extent with mitochondria in HeLa cells treated with U0126 and monitored by either optical (Fig. 2A) or confocal laser (Fig. S1D) microscopy. To exclude the possibility that this ‘unconventional’ localization of HIF-1α was caused by non-specific effects of U0126, hypoxia-induced HIF-1α was localized in HeLa cells cultured in the absence of fetal bovine serum (FBS), a condition that inactivates the ERK pathway. Serum withdrawal indeed caused inhibition of expression of VEGFA, P4HA1-1, BNIP3 and NDRG1, four HIF-1 target genes (Fig. 2B), cytoplasmic translocation of HIF-1α and its extensive colocalization with mitochondria (Fig. 2C). The mitochondrial localization of HIF-1α under serum deprivation was reversed to nuclear when cells were treated with Leptomycin B (LMB; Fig. 2C), a potent CRM1 inhibitor, suggesting that targeting of HIF-1α to mitochondria was due to stimulation of HIF-1α nuclear export following ERK inactivation. To confirm the results in a non-cancerous cell line, endogenous HIF-1α was also localized in primary rabbit tracheal smooth muscle (RTSM) cells. Under hypoxia, HIF-1α was predominantly nuclear but a minor fraction was readily detectable in cytoplasmic structures (Fig. 2D). However, upon serum withdrawal, a large pool of HIF-1α translocated to the cytoplasm and colocalized extensively with mitochondria, showing that mitochondrial localization of unmodified HIF-1α is not a unique property of cancer cells. In both HeLa and RTSM cell lines, colocalization of HIF-1α with mitochondria under ERK inactivation conditions occurred in all cells (Fig. S2). Furthermore, visual, quantitative and statistical evaluation (Fig. S2), clearly showed that HIF-1α colocalization with mitochondria was non-random, increased significantly after ERK inactivation and involved a large proportion (close to 50%) of the total cellular HIF-1α population.

To independently verify the ERK-controlled mitochondrial localization of HIF-1α, we examined the involvement of its known ERK-modification sites at Ser641 and Ser643 by analyzing under normoxia HeLa cells not expressing endogenous HIF-1α but overexpressing GFP-tagged wild-type HIF-1α or its HIF-1α-SA mutant lacking the ERK target sites. Wild-type GFP–HIF-1α was predominantly nuclear but mutant GFP–HIF-1α-SA was excluded from the nucleus and largely colocalized with mitochondria (Fig. 3A; Fig. S1E). In agreement, treatment with U0126 triggered a substantial translocation of wild-type GFP–HIF-1α out of the nucleus and extensive colocalization with mitochondria under normoxia (Fig. 3A), suggesting that the distribution of HIF-1α between nucleus and mitochondria is a process controlled predominantly by ERK and does not require any additional hypoxia-induced mechanisms apart from HIF-1α stabilization. The same results were obtained when Flag-tagged forms of ETD were examined (Fig. 3B), further suggesting that an unmodified ETD, the part of HIF-1 that interacts with mortalin, mediates hypoxia-independent but ERK-dependent targeting of HIF-1α to mitochondria. The mitochondrial localization of GFP–HIF-1α-SA under normoxia was also confirmed by confocal laser microscopy in a second unrelated cancer line, human hepatoma HuH7 cells (Fig. 3C). When these same cells were grown under hypoxia, treatment with kaempferol, a dietary flavonoid that inhibits HIF-1α phosphorylation by ERK (Mylonis et al., 2010) caused translocation of endogenous HIF-1α outside the nucleus and substantial colocalization with mitochondria (Fig. 3D) confirming again the results obtained with HeLa cells and U0126 (see Fig. 2A). In contrast to the almost exclusive localization of GFP–HIF-1α-SA and Flag–ETD-SA outside the nucleus and in association with mitochondria, inhibition of ERK activation caused partial translocation of wild-type HIF-1α, which might be attributed to the substantial but incomplete ERK inactivation (see Figs 4A,B and 5C,D below).
Association of unmodified HIF-1α with mitochondria was then also examined after subcellular fractionation of HeLa cells. Upon hypoxic induction, HIF-1α was recovered mainly in nuclear fractions (Fig. 4A, lane 7; Fig. 4B, lane 9) with very little HIF-1α in mitochondrial fractions (Fig. 4A, lane 7; Fig. 4B, lane 9) and none in the cytosol (Fig. 4A, lane 1; Fig. 4B, lane 1). Upon instigating ERK inactivation (as detected by strong reduction of the phospho-ERK signal) either by U0126 (Fig. 4A, lanes 2, 5 and 8;
Fig. 4B, lanes 2, 6 and 10) or serum deprivation (Fig. 4A, lanes 3, 6 and 9), the nuclear pool of HIF-1α was decreased (Fig. 4A, lanes 8 and 9; Fig. 4B, lane 10) with a parallel increase of its mitochondrial pool (Fig. 4A, lanes 5 and 6; Fig. 4B, lane 6) and concomitant weak detection in the cytosol (Fig. 4A, lanes 2 and 3; Fig. 4B, lane 2). In all cases, mortalin was detected in the mitochondrial fractions (Fig. 4A, lanes 4, 5 and 6; Fig. 4B, lanes 5, 6). Quantification of the immunoblotting signals (Table S2) showed that, upon ERK inactivation, HIF-1α in the nuclear fraction was reduced by ∼60%. At the same time, HIF-1α was increased more than threefold in the mitochondrial fraction and twofold in the cytosolic one. These results are in agreement with the microscopic data and confirm that a relatively large fraction of nuclear HIF-1α migrates to the mitochondria when its phosphorylation by ERK is inhibited.

Depletion of mortalin using small interfering RNA (siRNA)-mediated silencing in HeLa cells grown under hypoxia and treated with U0126 (Fig. 4B, lanes 4, 8 and 12), largely removed unmodified HIF-1α from the mitochondrial fraction (Fig. 4B, compare lane 8 with lane 6) and simultaneously increased its cytosolic pool (Fig. 4B, compare lane 4 with lane 2), indicating that mortalin plays an important role in mediating targeting of HIF-1α to the mitochondria. To identify which compartment of the mitochondria HIF-1α associates with, cells were treated with increasing concentrations of digitonin, a procedure that sequentially exposes compartments of mitochondria. Both mitochondrial HIF-1α and mortalin were accessible to their antibodies at conditions that exposed Tom20, a component of the OMM, but left components of the mitochondrial intra-membrane space (cytochrome c) or matrix (HSP60, also known as HSPD1).
inaccessible (Fig. 4C). This suggests that HIF-1α associates through mortalin with the OMM.

ERK inactivation triggers apoptosis when HIF-1α is released from mitochondria

OMM-associated proteins play an important role in the events that lead to mitochondrial outer membrane permeabilization (MOMP), release of apoptogenic proteins, such as cytochrome c, to the cytosol and induction of apoptosis by caspase activation. To investigate the role of mitochondrial HIF-1α in this process, we monitored cellular proliferation, cell death, the presence of apoptogenic markers in subcellular fractions, caspase activity and integrity of the mitochondrial membrane potential ($\Delta \Psi_m$) of cells grown under hypoxia, and treated with U0126 or depleted of HIF-1α or both. Treatment with U0126 under either normoxia or hypoxia did not affect cellular proliferation (Fig. 5A; Fig. S3A) or cell death (Fig. 5B; Fig. S3B), nor induced apoptosis under hypoxia as judged by the absence of cytochrome c and activated cleaved caspase 3 (hereafter activated Casp3) from the cytosolic extracts (lane 2 in Fig. 5C and Fig. S3C), the integrity of nuclear PARP (Fig. S3C, lanes 6 and 10) and the normal $\Delta \Psi_m$ (Fig. 5F; Fig. S3F). It, however, caused HIF-1α translocation to the mitochondria and higher expression of the anti-apoptotic truncated form of VDAC1, VDAC1-ΔC (Fig. 5C, lane 6). In contrast, depletion of HIF-1α under hypoxia reduced cellular proliferation after 48 and 72 h (Fig. 5A), weakly increased cell death (Fig. 5B), lowered VDAC1-ΔC expression (Fig. 5C, lane 7) and induced apoptosis, as determined by the detection of cytochrome c and activated Casp3 in the cytosolic fractions (lane 3 in Fig. 5C and Fig. S3C; Fig. S3D), partial degradation of nuclear PARP (Fig. S3C, lanes 7 and 10) and
limited loss of ΔΨm (Fig. 5F; Fig. S3F). Strikingly, a combination of both treatments (U0126 addition and HIF-1α depletion) led to a further reduction of cellular proliferation that was observable at 72 h (Fig. 5A), a further increase in cell death that was evident at all time points (Fig. 5B) and a stronger induction of apoptosis as shown by increased amounts of cytosolic cytochrome c and activated Casp3.

Fig. 4. ERK-controlled and mortalin-mediated association of HIF-1α with the OMM. (A) Immunoblotting of subcellular fractions derived from HeLa cells, grown under hypoxia for 16 h and treated with or without 5 μM U0126 or subjected to serum withdrawal (−FBS), using the indicated antibodies. (B) Immunoblotting of subcellular fractions derived from HeLa cells transfected with control (−si Mort.) or mortalin siRNA (+si Mort.) and grown under hypoxia for 16 h with or without 5 μM U0126, using the same antibodies as in A. In both A and B, actin, Tom20 and ARNT are used as markers of the cytoplasmic, mitochondrial and nuclear fractions, respectively. (C) Immunofluorescence microscopy of HeLa cells, grown under hypoxia for 16 h in the presence of 5 μM U0126, fixed and then permeabilized by using the indicated concentrations of digitonin, using antibodies against the indicated proteins. Tom20 is a marker of the outer mitochondrial membrane, cytochrome c of the intermembrane space and Hsp60 of the mitochondrial matrix. Scale bars: 10 μm.
Fig. 5. Mitochondrial HIF-1α inhibits apoptosis when ERK is inactivated. (A,B) Determination of cell proliferation (A) and cell death (B) of HeLa cells transfected with control (ctr) or HIF-1α siRNA (si) and, at 24 h post transfection, incubated under hypoxia with or without 5 μM U0126 for the indicated time periods. Results are mean±s.e.m.; corresponding results under normoxia are shown in Fig. S3A,B (n=6). (C) Immunoblotting, with antibodies against the indicated proteins, of subcellular fractions derived from HeLa cells, transfected with control or HIF-1α siRNA and grown under hypoxia for 16 h with or without 5 μM U0126. (D) Immunoblotting of subcellular fractions derived from HeLa cells expressing Flag–ETD-SA or Flag alone grown and analyzed as in C. In C and D, white arrowheads point to VDAC1 and black arrowheads to VDAC1-ΔC. (E) Determination of caspase 3 and 7 activity in HeLa cells transfected with control or HIF-1α siRNA and, at 24 h post transfection, incubated for 24 h under normoxia or hypoxia with or without 5 μM U0126. Results are mean±s.e.m. (n=9). (F) Quantification of fluorescence intensity of either monomeric (gray bars, green signal, reflects low ΔΨm) or aggregated (black bars, red signal, high ΔΨm) JC-1 in HeLa cells transfected with control (~si HIF-1α) or HIF-1α siRNA (+si HIF-1α) and, at 24 h post transfection, incubated for 48 h under hypoxia with or without 5 μM U0126. Results are mean±s.e.m.; corresponding images are shown in Fig. S3F. A total of 100 cells were analyzed in each condition from two independent experiments. **P<0.01; ***P<0.001 (unpaired t-test).
association of ETD with mitochondria alone is not sufficient to doxorubicin-triggered apoptosis (Fig. 6C,I) suggesting that mitochondria did not protect normoxic cells from etoposide- or doxorubicin instead of etoposide (Fig. 6G,H).

Exactly the same results were obtained when cells were treated with mito-red or mito-tracker complexes containing their DNA-binding domain (Gal4-DBD) fused to wild-type or mutant forms of the C-terminal part of HIF-1α (HIF-1α amino acids 348–826). These proteins lack the N-terminal DNA-binding and PAS domains but possess GAL4-dependent transactivation ability (Mylonis et al., 2006). In this case, neither the wild-type nor the SE form of Gal4-DBD–HIF-1α–348–826 provided significant protection from etoposide- or doxorubicin-triggered apoptosis (Fig. 6E,K), as expected by their lack of HRE-dependent transcriptional activity. In sharp contrast, the SA form increased levels of VCAD1–ΔC, reduced activated Casp3 (Fig. 6D,J, lane 7) and decreased caspase 3 and 7 activity (Fig. 6E,K), thus showing that the anti-apoptotic function of unmodified HIF-1α resides in its C-terminal portion and is independent of HRE binding or heterodimerization with ARNT. Similar results were obtained after expression of GFP-tagged wild-type or mutant forms of a shorter C-terminal domain of HIF-1α (Fig. 6F,L). This shows that the observed effects are independent of the N-terminal tag, and maps the non-genomic anti-apoptotic function of HIF-1α to amino acids 575–826, which comprise the inhibitory and C-terminal transactivation domains (ID and C-TAD, respectively).

Mitochondrial HIF-1α associates with VCAD1 and HK-II

To address the mechanism underlying the anti-apoptotic function of mitochondrial HIF-1α, immunoprecipitates of HIF-1α from cytoplasmic (cytosol plus mitochondria) or nuclear extracts of cells grown under hypoxia (Fig. 7A, lanes 5 and 7) and treated with U0126 (Fig. 7A, lanes 6 and 8) were analyzed for the presence of proteins known to reside on the OMM and play a role in apoptosis. In addition to mortalin, HK-II associated with cytoplasmic but not nuclear HIF-1α (Fig. 7A, compare lane 5 with 7). Association of both proteins with HIF-1α became stronger upon U0126 treatment, which also revealed the presence of VCAD1 in the same cytoplasmic complexes (Fig. 7A, lane 6), whereas under all conditions nuclear HIF-1α complexes contained only ARNT (Fig. 7A, lanes 7 and 8).

When mortalin was immunoprecipitated from cells grown under normoxia, it associated with VCAD1 (in agreement with previous observations, see Introduction) but did not interact detectably with HK-II (Fig. 7B, lanes 7–9). Under hypoxia, the cytoplasmic mortalin complexes contained, in addition to VCAD1, HIF-1α and HK-II (Fig. 7C, lanes 7,8 and 9) and ERK inactivation strengthened the association of HIF-1α with the mortalin complex (Fig. 7C, compare lanes 8 and 9 with 7). Taken together, these data demonstrate association of HIF-1α with VCAD1 and HK-II, both regulating mitochondrial-mediated apoptosis. Furthermore, it is likely that mortalin links HIF-1α to VCAD1, whereas HIF-1α interacts directly with HK-II (given that mortalin binds to VCAD1 but not to HK-II in the absence of HIF-1α) and, therefore, HIF-1α might facilitate HK-II recruitment to the VCAD1–mortalin–HIF-1α complex on the OMM.

DISCUSSION

Our study reveals that HIF-1α can directly inhibit apoptosis by binding to proteins of the OMM. This role of HIF-1α is distinct from other known HIF-1α functions, and several lines of evidence suggest that it is independent of HIF-1α transcriptional activity but depends on ERK-mediated modification of Ser641 and/or Ser643 in the ETD of HIF-1α. First, association of a large fracture of cellular HIF-1α with the OMM and protection from apoptosis under hypoxia is promoted upon ERK inactivation either by pharmaceutical means or by serum withdrawal, conditions that drastically inhibit HIF-1 transcriptional activity. Second, both binding of HIF-1α to mitochondria and protection from apoptosis
Fig. 6. Mitochondrial HIF-1α inhibits drug-induced apoptosis under normoxia through its C-terminal 252-amino-acid long, domain. (A) Immunoblotting with antibodies against the indicated proteins, of total cell extracts from HeLa cells, expressing different GFP–HIF-1α forms or GFP alone (denoted Ctr in quantitative results) and treated, at 20 h post transfection, with 75 μM etoposide for 4 h, wt, wild type. (B) Determination of caspase 3 and 7 activity in HeLa cells treated as in A; results are mean±s.e.m. (n=6). (C) Determination of caspase 3 and 7 activity in HeLa cells expressing different Flag–ETD forms or Flag alone and treated, at 20 h post transfection, with 75 μM etoposide for 4 h (mean±s.e.m.; n=6). (D) Immunoblotting of HeLa cells expressing different Gal4-DBD–HIF-1α (348–826) forms or Gal4-DBD alone and treated as in A. (E) Determination of caspase 3 and 7 activity in HeLa cells treated as in D; results are mean±s.e.m. (n=6). (F) Determination of caspase 3 and 7 activity in HeLa cells expressing different GFP–HIF-1α–575-826 forms or GFP alone and treated, at 20 h post transfection, with 75 μM etoposide for 4 h; results are mean±s.e.m. (n=6). In A, D, G and J, white arrowheads point to VDAC1 and black ones to VDAC1-ΔC. In C and I, insets show immunoblotting analysis of corresponding samples with an anti-Flag antibody. *P<0.05; **P<0.01; ***P<0.001 (unpaired t-test).
The ID of HIF-1α (amino acids 576–785) was originally defined (Jiang et al., 1997) because it repressed the transcriptional activity of both its adjacent transactivation domains, N-TAD (amino acids 531–575) and C-TAD (amino acids 786–826). Our data can now explain this early observation by showing that the ID contains not only an ERK-dependent NES but also an ERK-dependent mortalin interaction site (ETD, amino acids 616–826). Our data can now explain this early observation by showing that the ID contains not only an ERK-dependent NES but also an ERK-dependent mortalin interaction site (ETD, amino acids 616–826). Our data can now explain this early observation by showing that the ID contains not only an ERK-dependent NES but also an ERK-dependent mortalin interaction site (ETD, amino acids 616–826).

The unconventional direct involvement of HIF-1α in mitochondrial-driven apoptosis shown in this work is not the first non-canonical HIF-1α function (i.e. a function independent of DNA binding and transcriptional activation). HIF-1α has been involved in the regulation of the Notch pathway by stimulating the catalytic activity of γ-secretase (the enzyme that cleaves Notch) through physical interaction with the γ-secretase complex (Gustafsson et al., 2005). Participation of HIF-1α in cell cycle regulation involves direct binding to Cdc6 and inhibition of the activation of the minichromosome maintenance (MCM) protein complex, which blocks initiation of DNA replication and induces cell cycle arrest under hypoxia (Hubbi et al., 2013). Therefore, it appears that the role of HIF-1α in mediating the cellular response to hypoxia expands further than transcriptional regulation and includes physical interaction with proteins in different cellular compartments in order to control diverse processes such as differentiation, cell cycle progression and apoptosis.

Several transcription factors have been found to be associated with mitochondria, including ATF2 and p53 (Green and Kroemer, 2009; Lau et al., 2012). Regulation of ATF2 translocation to mitochondria is analogous to that of HIF-1α but a different kinase is involved: phosphorylation of ATF2 by PKCe stimulates its nuclear accumulation and transcriptional activity, whereas inhibition of this phosphorylation triggers ATF2 nuclear export and binding of ATF2 to the OMM where, in contrast to HIF-1α, ATF2 induces apoptosis by disrupting the complex between VDAC1 and hexokinase I (Lau et al., 2012). The fraction of p53 that translocates to the OMM under stress conditions interacts instead with pro- and anti-apoptotic proteins of the Bcl-2 family and triggers apoptosis by inducing MOMP (Green and Kroemer, 2009). Like HIF-1α, p53 also...

under hypoxic and ERK-inactivating conditions are impaired by overexpressing a peptide comprising the phospho-deficient form of ETD (ETD-SA) that contains the mortalin interaction and mitochondrial-targeting site but cannot affect the transcriptional activity of nuclear endogenous HIF-1. Third, protection from drug-induced apoptosis under normoxia is stronger upon expression of the phospho-deficient mutant form of HIF-1α (HIF-1α-SA), which lacks transcriptional activity, is excluded from the nuclei and colocalizes with mitochondria. Finally, HIF-1α deletion mutants that lack the DNA-binding or heterodimerization domains but contain the ID and C-TAD offer similar protection as to that provided by HIF-1α-SA.
interacts with mortalin, but this interaction counteracts both its transcription-dependent and -independent apoptotic functions by inhibiting p53–Bax complex formation and sequestering p53 in the cytoplasm, respectively (Lu et al., 2011). Therefore, mortalin might exert a dual anti-apoptotic activity under hypoxia and in cancer cells by both restraining pro-apoptotic p53 and supporting anti-apoptotic HIF-1α.

To conclude, our data suggest that HIF-1α participates in the formation of a VDAC1–mortalin–HIF-1α–HK-II complex that stimulates production of anti-apoptotic VDAC1-AC, protects the integrity of ΔΨm, inhibits cytochrome c release and prevents caspase activation, thereby blocking induction of apoptosis and cell death under hypoxia. As formation of this complex does not require hypoxia-dependent gene expression, it can be formed as soon as HIF-1α is stabilized and in the range of a few minutes after the onset of hypoxia. Thus, the non-genomic function of HIF-1α revealed in this study might represent an ‘early’ mechanism that operates when cells first encounter hypoxia and counteracts the ensuing immediate mitochondrial stress (e.g. increased production of reactive oxygen species and reduced production of ATP) until the ‘late’ HIF-dependent stimulation of adaptive gene expression kicks in and takes over. This early pro-survival mechanism mediated directly by HIF-1α might also explain the requirement of HIF-1α for acute phase (early window) ischemia preconditioning that protects against ischemia–reperfusion injury (Cai et al., 2008), given that the duration of the brief preconditioning ischemic episodes might be incompatible with de novo protein synthesis of survival factors encoded by HIF-1-target genes. In support of this hypothesis, hypoxic preconditioning (cycles of hypoxia and reoxygenation) of cultured rat cardiac myocytes has been shown to cause increased recovery of HIF-1α in mitochondrial fractions, although the functional significance of this was not investigated (Rane et al., 2009).

Protection by mitochondrial-associated HIF-1α might be especially important for quiescent or non-proliferating cells in which the ERK signaling pathway is inactive (Fig. 7D,E). Activation of the ERK pathway leads to phosphorylation and subsequent inactivation of the key pro-apoptotic factor Bim (also known as BCL2L11) (Ewings et al., 2007), thus offering protection against apoptotic insults. Therefore, cells with suppressed ERK are likely to be more sensitive to apoptosis when exposed to low oxygen, which would create the need for fast HIF-1α-mediated assembly of a pro-survival complex on the OMM. By contrast, cells with active ERK are more resistant to apoptosis but their rapid proliferation might create a greater need for upregulation of metabolic genes mediated by nuclear and transcriptionally active HIF-1α. In summary, control of HIF-1α phosphorylation by ERK acts probably as a ‘rheostat’ that determines the distribution of HIF-1α between nucleus and mitochondria and, subsequently, the balance between long-term genomic and short-term non-genomic adaptation to hypoxia. As combating the adaptation of cancer cells to hypoxia is a goal in anti-cancer therapy, targeting this mechanism could be exploited for therapeutic interventions.

MATERIALS AND METHODS

Plasmids, antibodies and immunoblotting

Cloning of full-length HIF-1α and of HIF-1α 348–826 or their mutant SA and SE forms into mammalian expression vectors pEGFP-C1 or pBXGI as well as cloning of wild-type and SA forms of the HIF-1α ETD (amino acids 616–658) into pGEX-4T1 and pCMV2-Flag was as previously described (Mylonis et al., 2008, 2006). The cDNA corresponding to HIF-1α-ETD-SE was obtained by PCR using pEGFP-HIF-1α-SE as a template, and subcloned as a BamHI fragment into pGEX-4T1 and pCMV2-Flag vectors. To generate wild-type and mutant pEGFP-HIF-1α-575–826 forms, CDNA was obtained by PCR using the corresponding pEGFP-HIF-1α forms as a template, and sub-cloned as BamHI fragments into pEGFP-C1. The following antibodies were used: affinity-purified rabbit polyclonal antibody against HIF-1α (1:1000; Lyberopoulou et al., 2007); rabbit polyclonal antibodies against mortalin (sc-13967, 1:1000 dilution), TOM20 (sc-11415, 1:1000 dilution) and Gal4–DBD (sc-577, 1:500 dilution), mouse monoclonal antibody against C-terminus of PARP-1 (sc-8007, 1:5000 dilution) or goat polyclonal antibody against VDAC1 (sc-8828, 1:500 dilution) all from Santa Cruz Biotechnology (Dallas, TX); rabbit polyclonal antibodies against phospho-ERK1/2 (9101, 1:1000 dilution), ERK1/2 (9102, 1:1000 dilution), HSP60 (4870, 1:1000 dilution), caspase 3 (6662, 1:1000 dilution) and cytochrome c (4272, 1:500 dilution), rabbit monoclonal antibodies against HK-II (6867, 1:1000 dilution) and cleaved caspase-3 (9664, 1:500 dilution) and mouse monoclonal antibody against actin (3700, 1:5000 dilution) all from Cell Signalling (Danvers, MA); mouse monoclonal antibodies against HIF-1α (610959, 1:1000 dilution), ARNT (611079, 1:500 dilution) and phosphoserine (612547, 1:1000 dilution) from BD Biosciences (San Jose, CA); mouse monoclonal antibody against the N-terminus of PARP-1 (ALX-804-211, 1:5000 dilution) from Enzo Life Sciences (Farmingdale, NY); and rabbit polyclonal antibody against HIF-2α (NB100-122, 1:1000 dilution) from Novus Europe (Cambridge, UK) or against Flag (F4042, 1:10,000 dilution) from Sigma-Aldrich (St Louis, MO). Immunoblotting was performed as previously described (Mylonis et al., 2006). Western blot images were taken using an Uvitec Cambridge Chemiluminescence Imaging System equipped with Alliance Software (ver. 16.06) and quantified by Uviband Software (ver. 15.03) provided with the instrument (Uvitec Cambridge, Cambridge, UK).

Cell culture and transfection

HeLa and Huh7 cells (supplied by ATCC and checked for mycoplasma) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and rabbit tracheal smooth muscle (RTSM) cells produced and validated as previously described (Chachami et al., 2007) in DMEM with F-12 both supplemented with 10% fetal calf serum (FCS) and 100 U/ml penicillin-streptomycin (Biochrom, Berlin, Germany) in an IN VIVO2 workstation (Baker Ruskinn, Sanford, Maine). When required, cells were treated for 16–72 h with 5 μM U0126 or 50 μM kaempferol (both from Sigma-Aldrich), or dimethyl sulfoxide (Applichem, Darmstadt, Germany) as solvent control. For serum-withdrawal experiments, cells were kept in DMEM supplemented with 100 U/ml penicillin-streptomycin (Biochrom, Berlin, Germany) and treated, when required, with 20 ng/ml Leptomycin B (LMB; Sigma-Aldrich) for 4 h. To induce apoptosis, cells were treated for 4 h with 75 μM etoposide (Sigma-Aldrich), 10 μM doxorubicin or dimethyl sulfoxide as solvent control. Transient transfections were performed as described previously by using Turbofect (Thermo Fisher Scientific) reagent (Mylonis et al., 2006).

Protein purification and in vitro binding assays

GST–ETD and its mutant forms were expressed in E. coli and purified as previously described for GST–HIF-1α (Chachami et al., 2005). Approximately 10 μg of GST, GST–ETD or its mutant forms were immobilized on 25 μl glutathione–Sepharose beads and incubated with protein extracts (~1 mg of total protein) from HeLa cells lysed with HNMT buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1% Triton X-100, 0.5 mM dithiothreitol and 0.2 mM PMSF). Incubation was performed in HNMT buffer for 16 h at 4°C in a total volume of 0.5 ml. The beads were harvested and washed three times, and proteins bound to ETD forms were eluted by incubating the beads with 50 μl of TEV protease solution (1 μg diluted in Tris–HCl pH 8.0) for 16 h at 4°C to cleave the spacer sequence between GST and the ETDs. A fraction of the eluates (5 μl) was analyzed with SDS-PAGE and visualized by AgNO₃ staining or western blotting, whereas the rest was used for analysis by mass spectroscopy.

Immunoprecipitation

Immunoprecipitation experiments were performed as previously described (Mylonis et al., 2008). Briefly, HeLa cell total extracts or fractions were incubated for 16 h at 4°C in HNMT buffer (25 mM Hepes pH 7.5, 150 mM
NaCl, 2 mM MgCl₂, 1% Triton X-100, 0.5 mM dithiothreitol and 0.2 mM PMSF) with protein A beads supplemented with 1 μg of anti-HIF-1α or anti-mortalin polyclonal antibodies. Precipitates were eluted with Laemmli loading buffer and analyzed by SDS-PAGE and immunoblotting.

**Reporter gene assay and real-time PCR**

Reporter gene assays were performed as previously described (Mylonis et al., 2006). Briefly, at 24 h post transfection, cells were treated with 5 μM U0126 for 16 h and fixed for 5 min with 3% formaldehyde for 3% room temperature. 

**Fluorescence and immunofluorescence microscopy**

Fluorescence and immunofluorescence microscopy were performed as previously described (Mylonis et al., 2008). Briefly, cells grown on coverslips were fixed with 3% formaldehyde in PBS for 5 min, followed by permeabilisation with 0.1% Triton X-100 at 4°C, and incubated with BSA for 1 h at room temperature. 

**In situ proximity ligation assay**

The in situ proximity ligation assay (PLA) was performed as previously described for the HIF-1α and ARNT interaction (Kourti et al., 2015). After appropriate incubation under normoxia or hypoxia, HeLa cells grown on slides were fixed with 3% formaldehyde in PBS for 5 min, were permeabilized with PBS with 1% Triton X-100 for 15 min at 4°C, and incubated with anti-HIF-1α or anti-mortalin antibodies for 16 h at 4°C and processed using the Duolink® PLA® In Situ Detection Kit (Sigma-Aldrich). Slides were counterstained with DAPI (100 μg/ml) before mounting and observed as described above. PLA signals were digitally quantified using the ITCN tool of ImageJ (Byun et al., 2006).

**siRNA-mediated silencing**

HeLa cells were incubated in serum-free DMEM for 4 h with siRNAs targeting HIF-1α (10 nM; Qiagen, Venlo, Netherlands) and/or mortalin (20 nM; Santa Cruz Biotechnology, Dallas, TX). siRNA-mediated silencing was assessed by real-time PCR (qPCR) using the LightCycler® 96 System (Roche, Basel, Switzerland). The mRNAs encoding VEGFA, P4HA1, BNIP3, NDRG1 and ACTB were amplified using convenient primers (sequences available upon request). Each sample was assayed in triplicate for both target and internal control. Relative quantitative gene expression was calculated by using the ΔACT method.

**Cell proliferation, cell death and caspase 3 and 7 activity assays**

For cell proliferation and cell death assays, HeLa cells were seeded into 96-well plates (1000 or 2000 cells/well, respectively) and transfected with siRNAs targeting HIF-1α (10 nM; Qiagen Venlo, Netherlands) or non-targeting siRNA (10 nM; Qiagen Venlo, Netherlands). At 24 h post transfection, cells were treated with 5 μM U0126 or with DMSO as solvent control for the indicated periods under normoxic or hypoxic conditions. Cell proliferation was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay’ kit (Promega, Madison, WI) and values were normalized to those in control experiments in the absence of cells in 96-well plates supplied with culture medium and DMSO or U0126 alone. For the cell death assay, cells were cultured in DMEM supplied with 1% FBS, and cell death was determined using the LDH Cytotoxicity Detection kit (Takara Clontech, Mountain View, CA). To assess caspase 3 and 7 activities, HeLa cells were seeded into 96-well plates (2000 cells/well) and transfected with the indicated plasmids. At 24 h post transfection, cells were treated either with 75 μM etoposide or 10 μM doxorubicin, or with DMSO as solvent control for 4 h under normoxia. Caspase 3 and 7 activity was measured with the ‘Caspase-Glo® 3/7 Assay’ kit (Promega, Madison, WI).

**Mitochondrial membrane potential assay**

Mitochondrial membrane potential (∆Ψm) was monitored by using the JC-1 Mitochondrial Membrane Potential Assay Kit® (Cayman, Ann Arbor, MI). In healthy cells with high ∆Ψm, JC-1 forms aggregates that emit red fluorescence, whereas, in apoptotic cells with low ∆Ψm JC-1 remains in the monomeric form that emits green fluorescence. Briefly, HeLa cells were cultured on coverslips and transfected with siRNAs as described above. At 24 h post transfection, cells were treated with 5 μM U0126 or with DMSO as solvent control for 48 h under hypoxic conditions. JC-1 was applied for 30 min at 37°C and cells were directly observed by fluorescence microscopy. Quantification of both fluorescent signals (McCloy et al., 2014) involved drawing an outline around each cell, measuring the area, fluorescence intensity and adjacent background using the ImageJ software (v.1.51 g, NIH), and calculating total corrected cellular fluorescence as TCCF= integrated density – (area of selected cell×mean fluorescence of background).

**Tryptic digestion, LC-MS/MS analysis and database search**

Samples were digested with trypsin using the filter-aided sample preparation (FASP) digestion protocol (Wizniewski et al., 2009). Proteins were dissolved in 8 M urea, 100 mM Tris-HCl pH 8.6 (UB buffer) and added on top of a centrifugal filtering unit with a 10 kDa molecular mass cut-off (Sartorius, Goettingen, Germany). The filters were centrifuged at 12,000 g and washed twice with UB buffer. The proteins were reduced with 10 mM DTT, alkylated for 30 min in the dark with 0.05 mM iodoacetamide, washed for 20 min on ice and soluble cytoplasmic proteins were collected in the supernatant by centrifugation at 2000 g at 4°C (Andrews and Faller, 1991). To solubilize organellar-bound proteins (including mitochondria), the pellet was resuspended in 400 μl of hypotonic buffer supplemented with digitonin (100 μg/ml), incubated in ice for 30 min and centrifuged at 7000 g at 4°C (Holden and Horton, 2009). Finally, the pellet, comprising nuclei and cellular debris, was resuspended in 400 μl of HNMT buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1% Triton X-100, 0.5 mM dithiothreitol and 0.2 mM PMSF) and incubated on ice for 30 min to collect soluble nuclear proteins after centrifugation at 7000 g at 4°C. To produce crude cytosolic (cytosolic and mitochondria-bound proteins) and nuclear fractions the first step of fractionation was omitted, and cell pellet was resuspended directly in 400 μl of hypotonic buffer with 100 μg/ml of digitonin and the procedure continued as described above.

**Selective permeabilization of mitochondrial membranes**

HeLa cells were grown under hypoxia (1% O₂) in the presence of 5 μM U0126 for 16 h and fixed with 3% formaldehyde for 5 min at room temperature. Increasing digitonin concentrations (0–1.5 mg/ml) were used to permeabilize membranes (Otera et al., 2005) and cells were processed for immunofluorescence with the indicated antibodies.

**Selective permeabilization of mitochondrial membranes**

HeLa cells were grown under hypoxia (1% O₂) in the presence of 5 μM U0126 for 16 h and incubated under normoxia or hypoxia. Cells were harvested and luciferase was determined according to the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Total RNA from HeLa was isolated using the NucleoZol reagent (Macherey-Nagel, Duren, Germany) and cDNA was synthesized with the SensiFAST™ cDNA synthesis kit (Bioline, Luckenwalde, Germany). Real-time PCR was performed with KAPA SYBR FAST qPCR (Kapa Kapa Biosystems, Wilmington, MA, USA) in a LightCycler® 96 System (Roche, Basel, Switzerland). The mRNAs encoding BNIP3, BNIP1, BNIP2, BNIP4, BNIP5, NDRG1 and ACTB were amplified using convenient primers (sequences available upon request). Each sample was assayed in triplicate for both target and internal control. Relative quantitative gene expression was calculated by using the ΔACT method.
three times with 25 mM ammonium bicarbonate and finally subjected to an overnight tryptic digestion at 37°C with 0.5 μg Trypsin Gold MS grade (Promega, Madison, WI) in 25 mM ammonium bicarbonate. Digested peptides were eluted from the filter twice with 100 μl water and dried in a vacuum centrifuge (Thermo Fisher Scientific, Waltham, MA). The samples were reconstituted with 20 μl solution of 2% (v/v) acetonitrile and 0.1% (v/v) formic acid, sonicated in a water bath for 3 min and analyzed with LC-MS/MS using an LTQ Orbitrap XL. Mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray source as previously described (Pangou et al., 2016). Protein identification was performed using Proteome Discoverer 1.4 software (Thermo Fisher Scientific) equipped with a SEQUEST HT search engine (Pangou et al., 2016). Oxidation of methionine, deamidation of asparagine and glutamine, carbamyldimethylation of cysteine and acetylation of the protein N-terminus were used as variable modifications. The peptides were filtered according to their XCorr score versus charge state (XCorr: +2≥2.0 and +3≥2.5).

Statistical analysis
Statistical differences between two groups of data were assessed using the unpaired t-test in the GraphPad Prism version 5.04 software; P<0.05 was considered to be significant (*P<0.05; **P<0.01; ***P<0.001).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
I.M. and G.S. designed research; I.M., M.K., M.S. and G.P. performed research; I.M. (Faculty of Medicine, McGill University, Canada) for reading and commenting on the manuscript.

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Supplementary information
Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.195339.supplemental

References


Fig. S1. HIF-1α association with mortalin on mitochondria.  

(A) Soluble extracts (input) or HIF-1α immunoprecipitates (IP) of HeLa cells grown at 1% O₂ for 16 hours +/- 5 μM U0126 were analyzed by immunoblotting (WB) with indicated antibodies.  

(B, C) Soluble extracts (input) or Mortalin immunoprecipitates (IP) of HeLa cells grown at 1% O₂ for 16 hours +/- 5 μM U0126 were analyzed by immunoblotting (WB) with indicated antibodies.  

(D) HeLa cells grown under hypoxia for 16 hr without or with 5 μM U0126 were analyzed by confocal laser microscopy following treatment with an anti-HIF-1α polyclonal antibody and Mitotracker.  

(E) Normoxic HeLa cells expressing wild-type or its phospho-deficient mutant form GFP-HIF-1α-SA were examined by confocal laser microscopy. To visualize mitochondria cells were incubated with the Mitotracker fluorescent dye (red) for 30 min before fixation.
Fig. S2. Analysis of ERK-controlled endogenous HIF-1α co-localization with mitochondria.

(A) Immunofluorescence microscopy of HeLa cells incubated at 1% O₂ for 16 hours +/- 5 μM U0126 with anti-HIF-1α antibody. To visualize mitochondria cells were treated with Mitotracker (red). (B) HeLa cells kept at 1% O₂ for 16 hours +/- fetal bovine serum (FBS) or after -FBS/+Leptomycin B (LMB; 20 ng/ml) treatment for 4 hours were analysed as in (A). (C) RTSM cells were incubated under hypoxia for 16 hours +/- FBS and were analysed as in (A). In (A), (B) and (C), images were collected with a 40x objective (scale bar = 10 μm). Middle panels are scatterplots of pixel intensities of HIF-1α (green, y axis) and Mitotracker signal (red, x axis). Graphs depict the Pearson's correlation coefficient and Manders co-localization coefficient as measured in a total of 20 individual cells from two independent experiments in each condition ± s.e.m. (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).
Fig. S3. Mitochondrial HIF-1α protects from apoptosis

(A) Cell proliferation curves of HeLa cells transfected with control or HIF-1α siRNA and, 24 hours post-transfection, incubated under normoxia +/- 5 μM U0126 for the indicated time periods ± s.e.m. (n=6). (B) Cell death curves of HeLa cells transfected and treated as in (A) for the indicated time periods ± s.e.m. (n=6). (C) Immunoblotting of subcellular fractions derived from HeLa cells, transfected with control or HIF-1α siRNA and grown under hypoxia for 16 hours +/- 5 μM U0126, with antibodies against the indicated proteins (same experiment as in fig. 5C). (D, E) Graphs represent cyt c/actin (upper panels) or act. casp3/actin (lower panels) protein levels according to densitometric analysis of blots shown in figures 5C and D respectively (mean values of two independent experiments). (F) HeLa cells transfected with control or HIF-1α siRNA and, 24 hours post-transfection, incubated under hypoxia +/- 5 μM U0126 for 48 hours. Following that, to assess the integrity of ΔΨm, JC-1 reagent was applied for 30 min at 37°C and cells were observed by fluorescence microscopy.
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**Table S1. Identification of the 75 kDa protein binding to GST-ETD-SA as mortalin.** List of mortalin peptides identified after mass spectrometric analysis of the precipitated proteins in the pull down assay performed with GST-ETD-SA (shown in Figure 1A).
Table S2. Quantitation of HIF-1α from HeLa subcellular fractions. Densitometric analysis of HIF-1α blots shown in Figures 4A, B and 5C (mean value of four independent experiments ±s.e.m.). In each subcellular fraction HIF-1α was normalized with the respective loading control. No comparison is possible between different fractions.

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Table S3. Quantitation of HIF-1α from HeLa subcellular fractions expressing Flag-ETD SA. Densitometric analysis of HIF-1α blots shown in Figure 5D (mean value of two independent experiments). In each subcellular fraction HIF-1α was normalized with the respective loading control. No comparison is possible between different fractions.