We identified a novel human cDNA encoding a mitochondrial protein, MTP18 (mitochondrial protein, 18 kDa) as a transcriptional downstream target of phosphatidylinositol (PI) 3-kinase signaling. We demonstrate that MTP18 mRNA as well as protein expression is dependent on PI 3-kinase activity. Confocal microscopy and biochemical fractionation revealed a mitochondrial localization of MTP18. Loss-of-function analysis employing antisense molecules revealed that MTP18 is essential for cell viability in PC-3 and HaCaT cells. We show that knockdown of MTP18 protein level results in a cytochrome c release from mitochondria and consequently leads to apoptosis. In addition, HaCaT cells with reduced levels of MTP18 become more sensitive to apoptotic stimuli. This effect is accompanied by dramatic subcellular alterations. Reduction of MTP18 impairs mitochondrial morphology resulting in the formation of a highly interconnected mitochondrial reticulum in COS-7 cells. Conversely, overexpression of MTP18 induces a punctate morphology of mitochondria suggesting also a functional role of MTP18 in maintaining the mitochondrial integrity. Hence, our data indicate an unexpected connection of PI 3-kinase signaling, apoptosis and the morphology of mammalian mitochondria.

The phosphatidylinositol (PI) 3-kinase pathway has been extensively studied for its role in regulating cell growth, development, motility, adhesion, glucose transport, immune response, and survival (1). A chronic activation of the PI 3-kinase pathway contributes to tumorigenesis and metastasis (2). Activation of PI 3-kinase signaling and its downstream effector, the protein-serine/threonine kinase Akt, is considered to be one major signaling pathway by which survival factors prevent apoptosis (3, 4). Some reports suggest that chronically activated PI 3-kinase can protect tumor cells against various apoptotic stimuli (3).

The activity of members of the Bcl-2 family is regulated by the PI 3-kinase downstream effector, the protein-serine/threonine kinase Akt. Hyperactivation of Akt inhibits the pro-apoptotic activities of Bad, Bax, and Bid (5–7). Akt has also been reported to inhibit apoptosis by phosphorylation of several other proteins (8). However, it is possible that besides Akt other additional effectors downstream of PI 3-kinase exist which are responsible for mediating cell survival. For instance, it is unknown whether besides controlling the activity of the cell death machinery the expression of other integral mitochondrial specific proteins is directly dependent on PI 3-kinase signaling.

The release of several proapoptotic mitochondrial proteins from mitochondrial intermembrane space into the cytoplasm has been reported to be crucial for the onset of apoptosis (9). In addition, recent reports point to a change in mitochondrial morphology as a prerequisite for the release of these mitochondrial factors (10, 11). Even though the mitochondrial disintegration during the executing steps of apoptosis is established, it seems that the maintenance of the mitochondrial morphology is also important to ensure mitochondrial function and homeostasis. For example mitochondrial dysfunction itself has been linked to various diseases and cancer (12–14). However, despite of the identification of several proteins involved in maintaining mitochondrial morphology the regulation or the participating signal transduction pathways for these processes are still unknown.

Here we present the identification and characterization of a previously unknown novel downstream effector of PI 3-kinase signaling. The PI 3-kinase-dependent expression is demonstrated on mRNA and protein level. We show that this novel protein MTP18 (mitochondrial protein, 18 kDa) localized to mitochondria of mammalian cells. Loss-of-function studies using MTP18-specific antisense molecules reveal that this mitochondrial protein is essential for cell viability. Moreover, reduction of the MTP18 protein level induces the release of cytochrome c, activates the caspase cascade, and leads to cell death. Overexpression as well as loss-of-function experiments suggest a functional role of MTP18 in maintaining the integrity of the mitochondrial network.

EXPERIMENTAL PROCEDURES

Cell Culture—Human prostate carcinoma PC-3, HeLa, and COS-7 cells (American Type Culture Collection) were cultured as described (15–17). Human keratinocytes (HaCaT) (18) were obtained from P. Boukamp (Deutsches Krebsforschungszentrum, Germany) and cultured as described (19). GeneBloc transfections were carried out as described (17). MTP18 expression plasmids were transfected using Effectene™ (Qiagen) according to the manufacturer’s instructions.

Cell proliferation was measured using WST-1 reagent (Roche Applied Science). PC-3 cells (5 × 10⁵) in 200 µl of culture medium were seeded into 96-well plates. After incubation for 24 h, the cells were transfected with GeneBlocs or treated with LY294002 (day 0). At the end of treatment, 10:1 of WST-1 reagent were added into wells and incubated for 1 h and measured according to the manufacturer’s protocol using a SpectraMax 190 (Molecular Devices). Cell growth on matri gel matrix (BD Biosciences) was assayed according to Sternberger et al. (17) using PC-3 cells (75,000 cells per well). To induce apoptosis, HaCaT

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§ The abbreviations used are: PI, phosphatidylinositol; TNFα, tumor necrosis factor α; FACS, fluorescence-activated cell sorter; HA, hemagglutinin; GFP, green fluorescent protein; GB, GeneBloc; MM, mismatch; RT, reverse transcriptase; PARP, poly(ADP-ribose) polymerase.
cells (1 x 10^6 in 10-cm plates) were kept in 5 ml of phosphate-buffered saline and exposed to 10 or 50 mJ/cm² using the UBV 500 UV cross-linker (Hoefer Scientific Instruments). After radiation, phosphate-buffered saline was replaced by 10 ml of fresh medium. For TNFα-induced apoptosis, cells were incubated with 5 or 10 ng/ml TNFα (+10 μg/ml cycloheximide) with or without 2 h. FACS analysis was performed as described (19).

**Immunoblotting and Antibodies**—The preparation of cell extracts and immunoblot analysis was carried out as described (16). Isolation of a highly enriched mitochondrial fraction from the cytosolic fraction of PC-3 cells was performed using a mitochondria/cytosol fractionation kit (Active Motif). The determination of mitochondrial cytochrome c release was analyzed using a digitonin-based subcellular fractionation technique as described in Takaasawa and Tanuma (47).

Polycional antibodies against full-length MTP18 were generated by immunizing rabbits with recombinant MTP18 protein produced in *Escherichia coli* using pET19-b expression vector (Merck Biosciences GmbH). The murine monoclonal antibodies anti-p110α and anti-p85α have been described (16, 20). Rabbit polyclonal anti-Akt and anti-phospho-Akt (Ser-473) antibodies, as well as polyclonal antibodies specific for cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology. The murine monoclonal anti-Hsp60, monoclonal anti-Drp1/DLP1 antibody, and monoclonal anti-cytochrome c antibody (clone 6H2.B4) were purchased from BD Transduction Laboratories. The rabbit polyclonal anti-ETS-1 and p110β antibodies were obtained from Santa Cruz Biotechnology.

**Plasmids and Antisense Oligonucleotides (GeneBlocs)**—The nucleotide sequence of human MTP18 cDNA is deposited under GenBank™ accession number AAH46132. The coding regions of the MTP18 gene was amplified by PCR using gene-specific primers and the Expand Long Template PCR System kit (Roche Applied Science). The amplified product was inserted into the pCR4-TOPO cloning vector (Invitrogen). HA- and MYC-tagged MTP18 expression constructs were generated by subcloning the MTP18 cDNA into pCG-HA (XbaI/BamHI) for N-terminal HA- and pCG-MYC (XbaI/SmaI) for C-terminal MYC fusion, respectively. GFP-MTP18 and MTP18-GFP were generated by PCR and PCR products were subcloned using pcDNA-NT-GFP-TOPO and pcDNA-CT-GFP-TOPO vector system, respectively (Invitrogen).

The used GeneBloc (GB) represents the third generation of antisense oligonucleotides (described in Ref. 17). The GBs used in this study have the following sequences: GB p110α, acucaaaACGCTCTTTGuccaguu; mismatch (MM) p110α, acucaaaACGCTTGTGuccaguu; GB p110β, ggcuaaCTTCTTTGCuccucac; MM p110β, ggcuaaCTTCTTGCuccucag; GB Akt 1, gucuugATGTACTCCcuucgu; MM Akt 1, gucuugATGTACTCCcuucag; GB Akt 2, uacuugATGATCTCCcuucac; GB Akt 2, uacuugATGATCTCCcuucag; GB1 MTP18, ggcucuGAAGCCTTgcucuc; MM1 MTP18, ggcucuGAAGCCTTgcucuc; GB2 MTP18, aguacuCTTCTTTgccucac; GB2 MTP18, aguacuCTTCTTTgccucag. The respective mismatch positions in the MM control oligonucleotides are underlined.

**Northern Blot and RT-PCR (TaqMan)**—Human multiple tissue Northern blots were purchased from BD Biosciences Clontech. The blots were hybridized with radiolabeled full-length MTP18 probe (Rediprime II, Amersham Biosciences) according to manufacturer’s protocol. Blots were analyzed using a PhosphorImager (Amersham Biosciences).

**RNA was isolated and purified using the Invitrogen spin cell RNA mini kit (Invitrogen GmbH). Inhibition of MTP18 mRNA expression was detected by multiplex real-time RT-PCR as described previously (19).**

**Immunofluorescence Microscopy**—About 15–20 h after transfection, cells were fixed for 15 min at room temperature in 4% formaldehyde/phosphate-buffered saline. All subsequent steps for indirect immunofluorescence and MitoTracker Red (Molecular Probes) staining were carried out as described previously (21). Nocodazole (5 μM) treatment was performed as described (22). An LSM 510 META confocal microscope (Zeiss) was used for microscopy.

**RESULTS**

**Identification of a Novel PI 3-Kinase-dependent Target Gene**—To identify novel downstream drug targets in the PI 3-kinase pathway, we analyzed gene expression changes in Pten<sup>−/−</sup> PC-3 cells (human prostate cancer cells) after inhibition of PI 3-kinase activity with the small molecule inhibitor LY294002 (23). The ability of cells to grow on or in extracellular matrix is indicative for the invasive growth potential of cancer cells (23, 24). Since the phenotypic consequences of PI 3-kinase inhibition are more pronounced on cells grown on extracellular matrix when compared with cells grown on plastic, we carried out our gene expression profiling experiments with RNA derived from cells grown on this semisolid surface (Fig. 1A) (23).
The RNA derived from matched samples of PC-3 cells treated with or without the PI 3-kinase-specific inhibitor (LY294002) were used to perform a genome wide gene expression profiling (Affymetrix). This experimental approach led us to the identification of a series of known and novel PI 3-kinase-dependent mRNAs (for details, see Ref. 23). Here we present the identification and characterization of a novel PI 3-kinase-dependent target gene (GenBankTM accession number AAH46132). According to the DNA chip analysis the mRNA level was reduced in PC-3 cells treated with 10 μM LY294002 when compared with the Me₂SO vehicle treated control cells (Fig. 1B, upper panel). To confirm the DNA chip analysis we employed real-time PCR (TaqMan) on independently derived RNA preparations (Fig. 1B, lower panel). With both methods a similar time-dependent decrease of the AAH46132 mRNA was observed. The mRNA level of p110α, one catalytic subunit of PI 3-kinase, was not changed and was used to normalize mRNA amounts in both assays.

Having established the PI 3-kinase-dependent mRNA expression we cloned the full-length cDNA of AAH46132 and generated polyclonal antibodies against the recombinant protein. Fig. 2A shows the full-length cDNA including the open reading frame used for recombinant expression of the protein. We named this novel protein MTP18 (mitochondrial protein, 18 kDa) because of the mitochondrial localization and its predicted mass of 18 kDa (see below). A data base search revealed protein homologues in other metazoa (mouse, Caenorhabditis elegans, Drosophila) but not in unicellular organisms like yeast (Fig. 2B). So far none of these homologues has been characterized functionally. Northern blot analysis with AAH46132-specific probes on RNA derived from human tissues revealed a single 1.4-kb-long mRNA. The size is consistent with the full-length construct and suggests the absence of splice variants (Fig. 2C). The strongest expression level was observed in heart and skeletal muscle but transcripts were also detected in other tissues including prostate, trachea, pancreas, kidney, and liver. Lower mRNA expression was detected in brain, lung, spinal cord, bone marrow, spleen, and lymphatic cells.

MTP18 Colocalizes and Cofractionates with Mitochondria—
To demonstrate specificity of the anti-MTP18 polyclonal antibody we performed immunoblots with lysates derived from HeLa cells transiently transfected with different MTP18 expression constructs. Signals were observed with N-terminal HA-tagged, C-terminal Myc-tagged, and GFP fusion protein constructs indicating the specificity of the anti-MTP18 serum (Fig. 3A). More importantly endogenous MTP18 protein was also detected in whole cell lysates of HeLa cells with the anti-MTP18 antibody. The apparent size of 18 kDa matches the predicted molecular mass deduced from the MTP18 primary sequence (Fig. 3A).

To analyze the subcellular localization of MTP18 we performed immunoblot experiments on fractionated cell lysates (Fig. 3B). Fractionation of the cell lysates in cytoplasmic, nuclear, and mitochondrial subfractions revealed that MTP18 cofractionates with the mitochondrial protein Hsp60 (Fig. 3B, lane 4). To demonstrate the quality of the subcellular fractionation we used the catalytic subunit of PI 3-kinase p110α and the transcription factor ETS-1 as cytoplasmic (lane 2) and nuclear markers (lane 3), respectively. To confirm the mitochondrial localization of MTP18 in cells we performed confocal microscopy using the anti-MTP18 antiserum on HeLa cells transfected with MTP18 expression vectors (Fig. 3C, left panel). Cofractionation with MitoTracker dye revealed a colocalization of mitochondria and MTP18 in transfected HeLa cells (see merged pictures, Fig. 3C, right panel). Notably, N-terminal-tagged GFP-MTP18 fusion protein (Fig. 3C) as well as N-terminally truncated MTP18*Δ1–30 (data not shown) did localize to mitochondria suggesting that MTP18 lacks a mitochondrial presequence targeting signal. Taken together, these data indicate an intracellular localization of MTP18 to mitochondria in mammalian cells.

Protein Expression of MTP18 Is Dependent on PI 3-Kinase Signaling—Reduction of mRNA level after LY294002 treatment in PTEN-deficient PC-3 cells suggests that the expression of MTP18 is dependent on PI 3-kinase signaling. To demonstrate the PI 3-kinase-dependent protein expression we analyzed MTP18 protein level in PTEN-positive differentiation-competent HaCaT keratinoctyes in response to serum. Whole lysates of HaCaT cells grown in the absence or after stimulation with serum were analyzed by immunoblot (Fig. 4A). Serum deprivation led to time-dependent reduction of MTP18, whereas the mitochondrial protein Hsp60 and the PI 3-kinase subunits p110α, p85, and Akt1/2 were not affected. (Fig. 4A, compare lane 1 with lanes 2–5). Phosphorylation of Akt was also decreased in a time-dependent manner after serum deprivation. Interestingly, restimulation by adding fresh serum led to an increase of MTP18 in a time-dependent manner (Fig. 4A, lanes 6–9). Stimulation of Akt phosphorylation by serum addition was more rapid, which is consistent with the idea that Akt kinase is a direct downstream effector of PI 3-kinase.

To confirm the dependence of MTP18 protein expression on PI 3-kinase signaling, we employed the PI 3-kinase inhibitor LY294002 in experiments with PTEN-deficient PC-3 cells. Immunoblot analysis of PC-3 cell lysates prepared at different time points after LY294002 (10 μM) treatment showed a rapid decrease in Akt phosphorylation and a more delayed reduction of MTP18 protein expression (Fig. 4B).

To verify the results obtained with the small molecule PI 3-kinase inhibitor LY294002, we used our antisense gene silencing tools, GBs (third generation antisense molecules (17)) to inhibit gene expression of different members of the PI 3-kinase pathway. We have shown previously that reduction of the PI 3-kinase subunit p110β, but not p110α or Akt1/2, leads to an inhibition of invasive growth of HeLa cells on matrigel (15, 25). Similarly, GB-mediated knockdown of p110α, Akt1, and Akt2 did not reduce the growth of PC-3 cells on extracellular matrix, whereas inhibition of p110β decreased growth dramatically (Fig. 4C). We have verified the knockdown of the individual proteins and the status of Akt phosphorylation by immunoblot analysis in comparison with corresponding MM controls (Fig. 4D). With respect to MTP18 expression only reduction of the catalytic subunit p110β decreased the MTP18 protein level (Fig. 4D, lane 3). Neither GB-mediated p110α nor Akt1/2 inhibition reduced the MTP18 protein level. These data suggest that MTP18 protein expression as well as PC-3 cell growth on the extracellular matrix is dependent exclusively on the catalytic subunit p110β but not p110α or Akt1/2 (Fig. 4D, lanes 1, 5, 7, and 9). From these data we conclude that the expression of this novel mitochondrial protein is dependent on PI 3-kinase but not necessarily dependent on signaling via Akt.

Inhibition of MTP18 Protein Expression Leads to Reduced Cell Growth—Next, we wanted to analyze the loss-of-function phenotype of MTP18. We performed a screen to identify antisense molecules (GBs), which are potent in reducing MTP18 protein expression. Fig. 5A shows the result of a transfection experiment in PC-3 cells with two different GBs or their corresponding mismatch controls (four mismatches were introduced, see “Experimental Procedures.”) Transfection of both GBs reduced the MTP18 protein level significantly at a 15 nM concentration 48 h post-transfection (Fig. 5A, lanes 5 and 13). We used the mitochondrial protein Hsp60 as a control for assaying equal levels of mitochondrial proteins in the whole cell lysates. In parallel we analyzed whether the GB-trans-
Fig. 2. Characterization of the full-length MTP18 cDNA. A, nucleotide and deduced amino acid sequences of human MTP18 cDNA. Initiation (ATG) and stop (TGA) codons are shown in bold. The polyadenylation signal (ATTAAA) is underlined. B, comparison of human MTP18 (GenBank™ accession number AAH46132) with homologues from mouse (GenBank™ accession number AK006148), C. elegans (GenBank™ accession number T13C5.6), and Drosophila (GenBank™ accession number CG7772). Sequences were aligned using the ClustalW algorithm. Black-shaded amino acids indicate identical residues; gray-shaded amino acids indicate similar residues. C, expression of MTP18 mRNA in human tissues. A single transcript was detected at 1.4 kb by Northern blot analysis. Each lane contains 2 μg of poly(A⁺) RNA derived from the indicated tissues. Multiple tissue Northern blot membranes were purchased from Clontech.
fected PC-3 cells showed impaired growth on extracellular matrix or in plastic two-dimensional cultures, similar to PI 3-kinase catalytic subunit p110β (Fig. 4C). Independently of the growth conditions, we observed growth inhibition of PC-3 cells with reduced MTP18 protein level (Fig. 5B). These data suggest that MTP18 is an essential protein in PC-3 cells. The GB-mediated inhibition of cell proliferation was also demonstrated more quantitatively and over a longer time period in a WST-1 metabolic activity assay (Fig. 5C). Again, the transfection of MTP18-specific GBs, but not of the corresponding mismatches, led to a dramatically reduced WST signal in this assay (Fig. 5C). The GB-mediated inhibition was even more pronounced than the LY294002 treatment (10M in Me2SO).

To analyze whether the reduced cell growth was due to an inhibition of cell cycle progression or an increase in apoptosis, we performed a FACS analysis. Interestingly, we did not observe a dramatic difference in cell cycle progression in GB- or mismatch-transfected PC-3 cells (Fig. 5D). However, with both MTP18-specific GBs a significant increase in the subG1 DNA fraction was observed indicating an increase in nuclei with reduced DNA content. This result suggests that a reduction of MTP18 protein in PC-3 cells leads to an increase in DNA fragmentation.

Knockdown of MTP18 in HaCaT Cells—Since PC-3 cells represent a highly transformed cell line we studied MTP18 loss-of-function phenotypes in normal human keratinocytes (HaCaT) (18). Analogous to the growth inhibition of PC-3 cells knockdown of MTP18 in HaCaT cells led to a dramatic reduction in cell proliferation (Fig. 6A). In this cell system cell growth was not only stopped, but the cell number decreased over time after GB treatment. It is important to note that the mismatch containing GBs did not show an anti-proliferative effect demonstrating that the transfection condition are not affecting normal cell proliferation.

To verify the specific MTP18 protein knockdown, we performed an immunoblot analysis with lysates from GB-transfected cells. MTP18 protein knockdown was already observed 15 h post-transfection with both GBs (Fig. 6C, upper panel). No reduction in protein expression was observed with the mismatch controls. The mitochondrial protein Hsp60 or the cytoplasmic p110α served as loading control. As mentioned above, we observed a decrease in cell number in transfection experiments with MTP18-specific GB. FACS analysis with the transfected HaCaT cells showed similar results as obtained with PC-3 cells observing an increase in the subG1 fraction (Fig. 6B). The increase of cells in the subG1 fraction points again toward an apoptotic DNA fragmentation in cells with reduced MTP18 protein level. It is well established that cytochrome c release from mitochondria into cytoplasm activates caspases, and in turn activated caspase-3 cleaves the death substrate PARP (26). To address the question whether the inhibition of MTP18 protein can stimulate apoptosis, we performed immunoblot analysis with cleaved PARP and cytochrome c-specific antibodies on fractionated lysates derived from GB transfected
cells. We observed with both GBs an increase in cleaved PARP signal in the cytoplasmic fraction (Fig. 6C, lanes 4 and 12 and 14). As a positive control we used lysates from UV-treated HaCaT cells (Fig. 6C, lanes 8 and 16). Knockdown of MTP18 as well as UV treatment led also to the release of cytochrome c from the mitochondrial fraction into the cytoplasm demonstrating the onset of apoptosis. This result suggests that loss-of-function of MTP18 protein induces apoptosis similar to UV treatment in HaCaT cells.

Reduction of MTP18 Results in Increased Response to Apoptotic Stimuli—In the next step we wanted to analyze whether UV- or TNFα-induced apoptosis is altered in HaCaT cells with reduced MTP18 protein level. HaCaT cells were transfected with MTP18-specific GB or the corresponding mismatch-containing molecules. 24 h later the transfected and untreated cells (UT) were exposed to two different doses of UV (Fig. 7A) or treated with two different concentrations of TNFα (Fig. 7B). The activation of apoptosis was measured by immunoblot analysis detecting cleaved PARP. In cells with GB1- and GB2-mediated MTP18 protein reduction we observed with both UV doses and both TNFα concentrations an increase in cleaved PARP signal when compared with mismatch-, UV-, or even TNFα-only-treated cells (Fig. 7, lanes 5, 8, 14, and 17). We concluded from these data that HaCaT cells with reduced MTP18 protein levels are more susceptible to UV- or TNFα-induced apoptosis.

Fig. 4. MTP18 protein expression is dependent on PI 3-kinase signaling. A, MTP18 protein level changes in response to serum in HaCaT cells. HaCaT cells were exposed to serum-free medium and harvested at indicated time points (lanes 2–5). For serum-dependent induction of MTP18 expression, HaCaT cells were grown in serum-free medium for 35 h before addition of serum (lanes 6–9). Immunoblot with whole cell extracts was probed using the indicated antibodies. B, inhibition of PI 3-kinase activity in PC-3 cells using the chemical inhibitor LY294002 (LY) (10 μM) results in a decrease of endogenous MTP18 protein. Cell extracts were prepared at the indicated time points and subjected to immunoblotting using the indicated antibodies. Efficient inhibition of PI 3-kinase activity is indicated by loss of Akt phosphorylation. DMSO, Me2SO. C, reduction of p110β expression but not p110α or Akt1/2 leads to inhibition of PC-3 cell growth on matrigel. PC-3 cells were transfected for 48 h with the indicated GeneBloc (60 nM). One part of the cell samples was lysed for protein analysis (D), and the other set was seeded on matrigel. Images were taken 48 h after seeding (bar, 200 μm; representative pictures are shown). D, GB-mediated inhibition of the PI 3-kinase catalytic subunits, p110α and p110β, or Akt isoforms have different consequences on MTP18 expression. Whole cell extracts were analyzed by immunoblotting using the indicated antibodies.
Changes in MTP18 Expression Affect Mitochondrial Morphology—Loss of MTP18 function caused cytochrome c release and apoptosis in HaCaT cell pools. To investigate loss-of-function effect of this novel mitochondrial protein in its cellular context, we were interested in studying this effect on single cell level. COS-7 cells were transfected with two MTP18-specific GBs or the corresponding MM controls. Cells were analyzed 24 h after transfection by immunoblotting to confirm specific reduction of MTP18 protein in GB-transfected cells (Fig. 8A). The transfected COS-7 cells were analyzed by immunofluorescence using anti-cytochrome c antibody to assess mitochondrial shape and cytochrome c distribution. Cells transfected with MTP18-specific GBs, but not with their corresponding MM controls, showed release of cytochrome c (Fig. 8B). Prolonged reduction of MTP18 protein resulted finally in increased cell death (data not shown). Additionally, confocal microscopy of GB-transfected COS-7 cells revealed aberrant mitochondrial morphology in contrast to MM-transfected cells.

The observed aberrant mitochondrial morphology prompted us to study this alteration in mitochondrial shape in more detail; GB-transfected cells displayed perinuclear mitochondrial aggregates (Fig. 8C, upper left panel) instead of the dispersed and equal distribution of mitochondria observed in non-transfected or MM-transfected cells (Fig. 8C, lower left panel). Treatment of the transfected cells with nocodazole prior to fixation according to Smirnova et al. (22) allows us to unfurl these perinuclear mitochondrial aggregate and revealed the existence of a highly connected network of mitochondrial filaments in 43% of GB1-treated cells (GB2: 47%) (Fig. 8C, upper right panel) compared with the rod-like-shaped mitochondria observed in the MM control sample (Fig. 8C, lower right panel). In contrast, only 5% of MM1-treated cells (MM2: 6%) of trans-
fected cells exhibited a highly connected mitochondrial network. This microscopic analysis demonstrates that GB-mediated reduction of MTP18 results in a change of mitochondrial morphology and cytochrome c release in COS-7 cells as observed before by Western blot for HaCaT cells.

Mitochondrial dynamics is maintained by specific, mitochondrial morphology controlling GTPases. One of these GTPases, the Drp1 protein for example, regulates fragmentation of mitochondria (27). Depending on the fission state, Drp1 cycles from the cytoplasm to mitochondria to execute fission. Therefore, changes in the balanced state of mitochondrial morphology can be monitored by analyzing Drp1 distribution. We were interested in studying the effects of loss-of-function and overexpression of MTP18 on mitochondrial morphology with respect to Drp1 distribution by immunofluorescence. COS-7 cells were transfected with MTP18-specific GB or the MM controls for 24 h, treated with nocodazole, and analyzed using anti-Drp1 antibody and Mitotracker. In untreated COS-7 cells Drp1 was found to be evenly distributed throughout the cytoplasm and as distinct spots scattered along mitochondrial filaments including their tips (Fig. 9A). About 55% of GB1-transfected cells (GB2: 46%) with reduced MTP18 protein levels exhibit the characteristic aggregates of interconnected mitochondria and show cytoplasmic distribution of Drp1 but lack

**Fig. 6. MTP18 protein knockdown results in apoptosis in HaCaT cells.** A, MTP18 protein knockdown inhibits cell proliferation in human keratinocytes (HaCaT). Cells were transfected with a 60 nM concentration of either MTP18-specific GBs or their corresponding MM controls. Images were taken at indicated time points (bar, 500 μm). B, cell cycle analysis of HaCaT cells 35 h after transfection with either MTP18 specific GBs or MM. C, MTP18 protein knockdown induces cytochrome c release and cleavage of PARP. HaCaT cells were transfected with either 60 nM MTP18-specific GB or MM and harvested at the indicated time points. Whole cell lysates were separated into cytoplasmic and mitochondrial fraction and analyzed by immunoblot using the indicated antibodies. Anti-Hsp60 and anti-p110α antibodies served as loading controls for the corresponding fractions. cyt c, cytochrome c; UT, untreated cells.
mitochondrial associated Drp1 spots (Fig. 9B, upper panel). In contrast, MM-treated cells with normal mitochondrial morphology displayed cytoplasmic distributed Drp1 as well as mitochondrial associated Drp1 spots (Fig. 9B, lower panel). This result suggests that reduction in MTP18 protein affects the balance that regulates mitochondrial morphology as indicated by the observed changes in Drp1 distribution.

In comparison with the loss-of-function situation, we studied the effects on mitochondrial morphology after overexpression of MYC-tagged MTP18. For overexpression analysis, COS-7 cells were transfected with an MTP18-MYC expression construct for 18 h and analyzed by immunofluorescence using polyclonal anti-MTP18 and anti-Drp1 antibodies. MTP18-MYC-expressing cells were identified by immunofluorescence using an anti-MTP18 antibody that detects transiently expressed MTP18-MYC protein but not endogenous MTP18 on mitochondria. Interestingly, 90% of MTP18-MYC-expressing cells show aberrant punctuate mitochondrial morphology (Fig. 9C) in contrast to the rod-like structure observed in non-transfected cells (Fig. 9A). Unlike the loss-of-function situation, Drp1 was almost entirely associated with these punctuated mitochondria in the MTP18-MYC-overexpressing cells but did not completely colocalize with MTP18-MYC-labeled mitochondria. Cytoplasmic distribution of Drp1 as observed in untreated or GB-treated cells was below detection level. Thus, the overexpression experiment suggests that high levels of MTP18 give rise to a change of mitochondrial morphology. To confirm that punctuate MTP18-MYC staining reflects mitochondrial morphology rather than changes in MTP18-MYC protein distribution, MTP18-MYC-transfected cells were stained with anti-cytochrome c antibody to reveal mitochondrial shape. In MTP18-MYC-transfected cells the fusion protein completely colocalizes with cytochrome c without affecting its distribution (Fig. 9D).

As shown before mitochondria in those cells appear as dot-like structures unlike the filamentous shape in non-transfected cells. The punctuate pattern of mitochondria induced by MTP18-MYC and the observation of Drp1 association with this structure suggest that MTP18 overexpression results in mitochondrial fragmentation. Taken together, loss-of-function analysis along with the overexpression result imply that MTP18 participates in mitochondrial dynamics.

**DISCUSSION**

In this study we describe the discovery and characterization of the novel human mitochondrial protein, MTP18. Our analysis revealed that: 1) MTP18 expression is regulated by the PI 3-kinase pathway; 2) MTP18 is essential for cell viability and loss-of-function induces cell death; and 3) MTP18 function is associated with the organization of mitochondrial morphology.

PI 3-kinase is mainly activated by cell surface receptors such as receptor tyrosine kinases and integrins and controls a major signaling pathway implicated in regulation basic cellular processes. We have inhibited PI 3-kinase activity using different approaches, including serum deprivation, LY294002 treatment, or PI 3-kinase-specific antisense molecules. With all three approaches we observed a significant reduction in MTP18 protein or mRNA expression indicating that MTP18 is a transcriptional downstream target of PI 3-kinase signaling (Figs. 1 and 4). So far only one other mitochondrial protein, the mitochondrial ATP-dependent Lon protease, has been described to be transcriptionally regulated by PI 3-kinase (28).
Mitochondria are involved in a variety of cellular processes and are known to participate in control of apoptosis (9, 29). PI 3-kinase signaling is generally considered to act in an anti-apoptotic manner. For instance inhibition of pro-apoptotic Bcl family members by the PI 3-kinase downstream effector kinase Akt is one well characterized link between this signaling pathway and mitochondrial function. Our data on MTP18 regulation suggest that there might be an Akt-independent link between PI 3-kinase signaling and mitochondrial function. We demonstrate that PI 3-kinase-dependent expression of MTP18 is independent of Akt function in the PC-3 cell system (Fig. 4). Likewise, we did not observe growth inhibition of PC-3 or HeLa cells on extracellular matrix after inhibition of Akt (Fig. 4C) (15). This is in agreement with previous reports indicating that Akt may be responsible for mediating some, but not all PI 3-kinase induced responses. For example, activated forms of Akt were found to be less potent in inducing proliferative and invasive cellular responses than the corresponding activated forms of p110 (16, 30, 31).

Inhibition of MTP18 expression leads to apoptosis indicated by the release of cytochrome c from mitochondria as shown in both HaCaT (Fig. 6C) and COS-7 cells (Fig. 8B). The MTP18 knockdown-dependent cytochrome c release activates the caspase cascade shown by detection of cleaved PARP, a caspase-3 cleavage substrate, and degradation of genomic DNA (Fig. 6, B and C). However, at this point it is not clear whether the cytochrome c release is a direct or indirect consequence of MTP18 loss-of-function. For instance, the induced mitochondrial disintegration may lead to a passive cytochrome c release, or the loss of MTP function initiates directly the activation of pro-apoptotic proteins such as Bax. The exact mechanism and processes leading to cytochrome c release and apoptosis in cells with reduced MTP18 protein has to be elucidated.

In addition, we provide evidence that MTP18 might be in-

**Fig. 8. Reduction of MTP18 affects mitochondrial morphology and induces cytochrome c release.** A, Western blot on whole cell lysates from COS-7 cells transfected with MTP18-specific GBs and their corresponding MM controls using MTP18-specific antiserum. Detection of Hsp60 served as a loading control. B, confocal microscopy analysis of COS-7 cells treated with MTP18-specific GB and stained with anti-cytochrome c antibody. Arrow, GB-treated cells showing cytochrome c release. Quantification of COS-7 cells exhibiting cytochrome c release 25 h after transfection with MTP18-specific GBs and their corresponding MM controls (150 cells counted). C, confocal microscopy of COS-7 cells transfected with either MTP18-specific GB1 (upper row) or MM1 (lower row) and stained with anti-cytochrome c. Cells (overview and corresponding close-up view) treated with nocodazole (5 μM for 1 h, right panels) and without treatment (left panels) are shown.
volved in the maintenance of mitochondrial morphology. MTP18 loss-of-function appears to promote fusion of mitochondria whereas transient overexpression of MTP18 causes mitochondrial fission (Figs. 8 and 9). These data indicate that the level of MTP18 protein expression affects mitochondrial dynamics. In cultured cells, mitochondria are dynamic and pleomorphic organelles that undergo balanced fission and fusion events to maintain their morphology (32). Considerable progress has been made recently in the understanding of the molecular mechanism of mitochondrial fission and fusion. Con-

**Fig. 9.** Knockdown and overexpression of MTP18 lead to changes in mitochondrial morphology and affects Drp1 distribution. A, COS-7 cells stained with MitoTracker (red) for visualizing mitochondrial morphology and anti-Drp1 (green) to analyze Drp1 distribution. Arrows, Drp1 protein associated with mitochondria in close-up view (right panel). B, COS-7 cells transfected with either MTP18-specific GB (upper row) or MM (lower row) and treated with nocodazole were stained with Mitotracker (red) and counterstained with anti-Drp1 antibody (green). Arrows, mitochondrial localization of Drp1 in the MM control transfected cell (right lower close-up panel) but not in GB-transfected cell (right upper close-up panel). C and D, microscopic analysis of COS-7 cells transfected with MTP18-MYC expression construct. MTP18-MYC expression was shown by using anti-MTP18 antibody (red); Drp1 distribution (green) was analyzed using anti-Drp1 antibody (C) or counterstained with anti-cytochrome c antibody (green) (D).
MTP18 Is Implicated in Mitochondrial Dynamics

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