Tim50, a Component of the Mitochondrial Translocator, Regulates Mitochondrial Integrity and Cell Death*

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In yeast, Tim50 along with Tim23 regulate translocation of presequence-containing proteins across the mitochondrial inner membrane. Here, we describe the identification and characterization of a novel human mitochondrial inner membrane protein homologous to the yeast Tim50. We demonstrate that human Tim50 possesses phosphatase activity and is present in a complex with human Tim23. Down-regulation of human Tim50 expression by RNA interference increases the sensitivity of human cell lines to death stimuli by accelerating the release of cytochrome c from the mitochondria. Furthermore, injection of Tim50-specific morpholino antisense oligonucleotides during early zebrafish embryonic development causes neurodegeneration, dysmorphic hearts, and reduced motility as a result of increased cell death. These observations indicate that loss of Tim50 in vertebrates causes mitochondrial membrane permeabilization and dysfunction followed by cytoplasmic release of cytochrome c along with other mitochondrial inducers of cell death. Thus Tim50 is important for both mitochondrial function and early neuronal development.

The mitochondrion is the bioenergetic and metabolic center of eukaryotic cells that also serves to amplify apoptotic signals. This essential organelle consists of four compartments, the matrix, the inner membrane, the inter membrane space and the outer membrane. The matrix is surrounded by the inner mitochondrial membrane and the inter membrane space is the compartment between the inner and the outer mitochondrial membranes (1). Many cellular stress signals induce permeabilization of the outer mitochondrial membrane resulting in the release of several mitochondrial proteins, which normally reside in the inter membrane space of mitochondria, into the cytosol and/or the nucleus (2–4). These released proteins are capable of activating the cellular apoptotic programs directly by activating caspases, cleaving nuclear DNA and neutralizing cytosolic inhibitor of apoptosis proteins (IAPs)1 (3, 5). Among the released proteins, cytochrome c can bind to the WD-40 repeats domain of Apaf-1 (apoptotic protease-activating factor 1) and increases its affinity to dATP/ATP. The binding of dATP/ATP to Apaf-1 triggers its oligomerization and recruitment of procaspase-9 to this complex, which is known as the apoptosome (6–8). The apoptosome facilitates autoactivation of caspase-9, which subsequently cleaves and activates downstream executioner caspases, such as caspase-3 (9).

Recent studies in mammalian cells have uncovered other mitochondrial proteins, such as Smac (Dia1lo) (10, 11) and the serine protease Omi (HtrA2) (12–15), whose release from the mitochondria contributes to apoptosis. Both Smac and Omi can bind to IAPs via a conserved N-terminal IAP binding motif (16). Interestingly a similar IAP binding motif is present at the N terminus of the small subunit of mature caspase-9 (17), which is necessary for inhibition of caspase-9 by IAPs. Because of their ability to bind to IAPs, Smac and Omi could disrupt the caspase-IAP complexes thus freeing the active caspases to induce apoptosis (17). Moreover, Omi has been recently shown to directly degrade IAPs in human cells (18, 19). Other mitochondrial proteins such as AIF (apoptosis-inducing factor) and endonuclease G (Endo G) are also specifically released from the mitochondria and directly targeted to the nucleus causing chromatin condensation and large scale DNA fragmentation (20, 21).

Most mitochondrial proteins are encoded by nuclear genes, translated in the cytosol as precursor proteins and subsequently directed to the mitochondria. The mitochondrial precursor proteins are recognized and sorted to different mitochondrial compartments by specialized translocator complexes in the outer and inner mitochondrial membranes (22, 23). Three translocator complexes have been identified in the mitochondria of Saccharomyces cerevisiae (24). The Tom (translo- case of the outer mitochondrial membrane) complex in the outer membrane recognizes mitochondrial precursor proteins and facilitates their entry through the outer mitochondrial membrane (25). The Tim (translo- case of inner mitochondrial membrane) complexes, Tim23 and Tim22, mediate translo- case across and insertion of precursor proteins into the inner membrane (26–28). Recent studies in yeast demonstrated that translocation through the Tom complex is linked to translocation through the Tim23 complex via a protein called Tim50 (29–31). Tim50 is a subunit of the Tim23 complex that appears to function as a mitochondrial pore opening protease, such as Smac and Omi. This illustrates the importance of Tim50 in maintaining mitochondrial integrity and cell survival.

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1 The abbreviations used are: IAPs, inhibitors of apoptosis; MTS, mitochondrial targeting sequence; TM, transmembrane; CTD, C-terminal domain; CPD, CTD-like phosphatase domain; GST, glutathione-S-transferase; PBS, phosphate-buffered saline; TRAIL, TNFα-related apoptosis-inducing ligand; Z, benzoxycarbonyl; FMK, fluoromethyl ketone; CNS, central nervous system; MO, morpholinio; Tim, translo- case of inner mitochondrial membrane; PK, proteasome K; hpf, hours postfertilization; dpf, days postfertilization; Tom, translo- case of the outer mitochondrial membrane; IMS, intermembrane space; pNP, p-nitrophenol; COX, cytochrome c oxidase subunit II; pnPP, p-nitrophenylphospho- phate; TNF, tumor necrosis factor; GFP, green fluorescent protein.
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to function as a receptor for the presequence-containing precursor proteins while in transit in the intermembrane space (IMS), and facilitates their transfer from the Tom to the Tim23 complex.

Few studies have addressed the role of components of the mitochondrial translocase in the regulation of the mitochondrial apoptotic pathway or the release of mitochondrial apoptotic proteins in mammalian cells. Only one study reported that insertion of the antiapoptotic protein Bcl-2 in the outer mitochondrial membrane requires interactions with components of the mitochondrial Tom complex (32). In this report we describe the identification and characterization of a novel human mitochondrial protein homologous to the recently identified yeast Tim50. We demonstrate that the human protein, designated human Tim50, is present in a complex with the Tim23 translocase. RNA interference-mediated knockdown of human Tim50 expression in cultured human cells resulted in accelerated cytochrome c release and apoptosis. Moreover, suppression of the translation of the zebrafish Tim50 ortholog by morpholino (MO) antisense oligonucleotides (33, 34) resulted in a variety of developmental defects that included pronounced neurodegeneration. Our results indicate that Tim50 is essential for maintaining the integrity of the outer mitochondrial membrane and for normal development of a model vertebrate.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid cDNA Library Screening and cDNA Cloning of the 40 kDa Protein—The yeast two-hybrid screening was performed essentially as described in the display GREEN-BASIC two-hybrid system protocol (Display Systems Biotech). Among 1.3 × 10^7 library (MCF7-Fas cells) plasmids screened, 36 interacting clones were obtained. Transformants were scored for activation of LEU and GFP reporter genes under the control of lexA operator. Plasmid combinations that resulted in growth on leucine-deficient medium within 4 days were considered positive. Expression of GFP in these growing colonies was confirmed by visualization under UV illumination.

The full-length 40 kDa protein cDNA was cloned from a human Jurkat Uni-ZAP XR cDNA library by PCR. This was done using primers corresponding to the 3′-region of the target gene as antisense primers and vector-specific primers as sense primers.

Expression Vectors and Antibodies—Constructs encoding full-length 40 kDa protein or truncated mutants were generated by PCR using modified complementary PCR adapter primers. FLAG and T7 epitope tagging was done by cloning the PCR-generated cDNAs of the respective genes in-frame into pFLAG CMV-2 (IBI Kodak) and pcDNA3-T7 (Invitrogen) vectors, respectively. Plasmids encoding GFP fusions were constructed using pEGFP-N1 (Clontech). Full-length 40 kDa protein and its mitochondrial targeting sequence (MTS) (residues 1–50) and CTR (residues 110–353) were overexpressed in Escherichia coli strain DH5α as N-terminally GST-tagged proteins using a pGEX2T vector (Amersham Biosciences). A polyclonal antibody against peptides of the 40 kDa protein (amino acids 293–306) was generated by Research Genetics, Inc. Anti-citrate synthase polyclonal antibody is a gift from Dr. Yuri Lazebnik. Anti-Omi monoclonal antibody was raised against mature Omi. Antibodies purchased from commercial sources are as follows: anti-Cyt c antibody (BD PharMingen), anti-Bax antibody (Upstate Biotechnology), anti-human cytochrome c oxidase subunit II (A-6404) (Molecular Probes), anti-Tim23 (BD transduction), anti-Tom22 (Sigma). Protein concentrations were determined by the Bio-Rad assay kit.

Transfection, Immunoprecipitation, and Immunoblot Analysis—293T cells (6 × 10^5 cells) in 100-mm dishes were transiently transfected

FIG. 1. Sequence and tissue distribution of the 40 kDa DR5-interacting protein. A, colinear alignment of the deduced protein sequences of the human and mouse 40 kDa protein. The peptide sequence (residues 293–306) used for generating the 40 kDa protein antibody is underlined. The mitochondrial import signal sequence (residues 1–31) is marked by an arrow. The percent similarity and identity between the human and mouse 40 kDa proteins are 96 and 93%, respectively. B, distribution of the 40 kDa protein in various human tissues. Protein samples (150 μg of total protein/sample) of various human tissues (Clontech) were fractionated on 12.5% SDS-polyacrylamide gel, and then probed with the 40 kDa protein antibody, preimmune serum, or Omi antibody. The last two antibodies were used as controls to ascertain the specificity of the 40 kDa antibody and the relative abundance of the 40 kDa protein in the tested tissue samples, respectively.
with the expression plasmids using the LipofectAMINE™ (Invitrogen) method as per the manufacturer's instructions. 24 h after transfection, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl containing 0.5% Nonidet P-40, 10 μg/ml leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation at 15,000 × g for 15 min. The clarified lysates were preabsorbed on protein G-Sepharose (Amersham Biosciences) and then incubated with anti-FLAG-M2 monoclonal antibody (Eastman Kodak Co.) for 2 h, followed by protein G-Sepharose beads. Immune complexes were washed extensively in the lysis buffer and eluted by boiling in SDS sample buffer. The eluted proteins were resolved by SDS-PAGE and detected by Western blot analysis with a horseradish peroxidase-conjugated T7 antibody (Novagen).

**Phosphatase Assay**—Reaction mixtures (100 μl) containing 50 mM Tris acetate (pH 5.5), 10 mM pNPP (p-nitrophenylphosphate), and human Tim50 or human Tim50 mutants as specified were incubated for 30 min at 37 °C. The reactions were quenched by adding 900 μl of 1 M sodium carbonate. Release of p-nitrophenol (pNP) was determined by measuring A410 and extrapolated from a pNP standard curve (35).

Protein-tyrosine phosphatase activity of human Tim50 proteins were measured against phosphotyrosyl peptides, END(pY)INASL (PS1) and DADE(pY)LIPQQG (PS2), according to the manufacturer's instructions (Promega, Madison, WI). Protein serine/threonine phosphatase activity was measured against phosphothreonyl peptides, RRA(pT)VA, using the serine/threonine assay system (Promega), according to the manufacturer's instructions.

**Subcellular Fractionation**—Cells were homogenized in buffer A (20 mM HEPES, pH 7.5, 10 mM MgCl2, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, with protease inhibitor mix). The homogenate was centrifuged at 800 × g. Nuclei were prepared from the crude nuclear pellet. The supernatant was centrifuged at 10,000 × g, and the resultant pellet was further processed for mitochondria purification over a Percoll gradient (36). Cytosol and microsome fractions were separated at 100,000 × g.

**Limited Trypsin or Proteinase K (PK) Digestion of Mitochondrial Fractions**—Isolated mitochondria from HeLa or 293T transfected cells were incubated on ice for 30 min in buffer A containing trypsin or PK at the indicated concentrations. At the end of the incubation period, digestion was stopped by adding 100 μg/ml soybean trypsin inhibitor, or 2 mM phenylmethylsulfonyl fluoride. Each sample was centrifuged at 12,000 × g to pellet mitochondria. Equal volumes of each sample were then separated by SDS-PAGE and immunoblotted with the indicated antibodies (37). For the generation of mitoplasts, purified mitochondria were diluted 10-fold with 20 mM HEPES/KOH (pH 7.2) followed by freeze-thawing three times in liquid N2 and 37 °C water bath.

**Immunofluorescence Microscopy**—HeLa cells were grown on cover slips. After cells were fixed with 4% paraformaldehyde, cells were stained with a polyclonal antibody raised against the 40 kDa protein peptide (residues 293–306). Fluorescein isothiocyanate-conjugated antibody (Novagen). After staining, the cover slips were mounted on slides and observed using confocal microscopy.

**Preparation of siRNA**—Sense and antisense oligonucleotides corresponding to human Tim50 were purchased from Qiagen-Xeragon: CGAACCCTGCTGGAGCACU (nucleotides 882–901). The sense and antisense oligonucleotides were annealed following manufacturer’s protocol to generate the double-stranded siRNAs at the final concentration of 20 μM.

**Fig. 2. Primary structure of the 40 kDa protein and two related human proteins.** A, domain structures of the 40 kDa protein, HSPC129, Dullard homolog, and yeast Tim50 are represented by bar diagrams. The numbers indicate the boundaries of these domains. B, sequence alignments of the four proteins in A.
To construct the siRNA-expressing vectors, the siRNA expression cassette (designed according to instructions on katahdin.cshl.org:9331/RNAi/html/docs/Huh.pdf) was subcloned into pSHAG vector between the BseRI and BamHI sites (38, 39). The resulting plasmid was confirmed by DNA sequencing.

Transfection of siRNA and UV or Staurosporine Treatment—293T cells were cultured in 6-well plates in 2 ml of Dulbecco’s modified Eagle’s medium (DMEM)-F12 medium complemented with 10% fetal bovine serum and transfected at 40% confluency by adding 10 μl of oligofectamine (Invitrogen) and 10 μl of 20 μM siRNA (final concentration 100 nM) in Opti-MEM medium. After 4 h, DMEM-F12 medium containing 30% fetal bovine serum was added to the transfected cells to a final concentration of 10% fetal bovine serum, and the cells were maintained in culture for an additional 24 h. Cells were rinsed and split into dishes (for cytochrome c release assay) or 12-well plates with slides on the bottom (for annexin V apoptosis assay) at 4 × 10^5/ml and kept in culture for 48–72 h. Cells were then treated with UV irradiation or the indicated amount of staurosporine.

Permeating Cells by Digitonin and Estimation of Cytochrome c Release and Apoptosis—Cells were collected and washed twice with ice-cold PBS, pH 7.4, followed by one time wash with extracellular buffer (120 mM NaCl, 0.1 mM EGTA, 20 mM HEPES-NaOH, pH 7.4). Cells were then incubated on ice for 15 min with 80 μg/ml digitonin in intracellular buffer (120 mM KCl, 10 mM NaCl, 1 mM KH2PO4, 20 mM HEPES, 1 mM dithiothreitol, and protease inhibitors complete mixture, Roche Applied Science). Approximately, 1 × 10^6 cells were diluted in 100 μl of buffer containing digitonin. Cell homogenates were spun at 14,000 rpm for 10 min, and supernatants I were removed and stored at −80 °C. 80 μg of supernatant II were loaded for Western blotting with COX antibody and human Tim50 antibody.

Apoptosis was quantified by phosphatidylserine exposure and monitored through characteristic changes in cellular morphology.

Identification of Human Tim50

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External-
ization of phosphatidylserine was detected by annexin V-fluorescein isothiocyanate staining and apoptotic cells were counted by fluorescence microscopy, as described in the instruction manual (BD Biosciences).

Embryos and Morpholino Oligonucleotides Injections—Fish stocks were raised under standard laboratory conditions (40). Embryos were incubated at 28.5 °C and staged in hours postfertilization (hpf) and days postfertilization (dpf). Morpholinos were designed with sequences complementary to zebrafish Tim50 based on the company’s recommendations (Gene-tools). The zebrafish Tim50 morpholino sequences were: MO 1. 5'-TGGGATACACAGACACCGCCGACAT-3' and MO 2. 5'-CG-GCTCGCAGCACACATGGGAT-3'.

Morpholino solution (5 pmol/embryo) was injected into the blastomere of each embryo at the 1–4 cell stage. As phenol red has no effect on zebrafish development, it was used as control or mixed into each sample to monitor the success of injection and distribution of morpholino in the embryos. Silent two-point-mutated zebrafish Tim50 was subcloned into the vector and transfected using the S6 Message Machine Kit (Ambion). About 1 nl of 210 ng/µl in vitro synthesized mRNA was microinjected into one-cell stage for the rescue experiments.

Whole Mount in situ Hybridization and Immunostaining—Digoxigenin-labeled antisense RNA probes were synthesized from full-length cDNAs using an in vitro transcription kit (Promega). As a control, sense RNA-labeled probes were synthesized and used for hybridization as above. Hybridization and development of whole mount zebrafish embryos were performed as described (41, 42).

For immunostaining, embryos were collected at the appropriate stage and fixed in 4% paraformaldehyde, pH 7.0, in PBS, overnight at 4 °C. Fixed embryos were dechorionated, washed three times with PBS, and stored in methanol at -20 °C. Znp-1 and F-59 antibodies are generous gifts from Dr. Michael Granato. Immunostaining was performed as described (43).

Acridine Orange Staining Assay—Embryos were dechorionated manually and placed in equal amount of heptane and 0.05 µg/ml acridine orange in PBS buffer. After 5 min of shaking, embryos were removed from the interface and rinsed three times (5 min each) with PBS. Samples were viewed with a conventional fluorescence microscope.

Photography and Image Processing—For photography, whole mount in situ hybridized embryos were postfixed in 4% paraformaldehyde, washed three times with PBS, dehydrated with methanol, cleared in methyl salicylate and mounted onto a glass slide with Permount. Living embryos of the desired stage were manually dechorionated and anesthetized in 0.03% tricaine (Sigma). The embryos were immobilized in 2% methylcellulose and photographed using differential interference contrast optics. Pictures were photographed on an Axioplan microscope (Zeiss) using digital camera. Photo images were cropped and assembled using the PhotoShop program (Adobe).

RESULTS

Identification and Cloning of a Human 40 kDa DR5-interacting Protein—Signaling by the cell membrane TRAIL receptors, DR4 and DR5, is mediated by their conserved cytoplasmic death domains, which recruit proteins that mediate a variety of cellular responses including cell death and proliferation. We performed a yeast two-hybrid screen to identify human proteins that interact with the death domain of DR5. Among the isolated cDNA clones, one was able to confer on yeast the ability to grow on Leu-minus medium and strong activation of the GFP reporter gene in the presence of the DR5-death domain bait (data not shown). This cDNA clone was found to encode a novel N-terminal truncated protein of unknown function (Fig. 1A, residues 5–353). The complete cDNA was isolated...
HeLa mitochondria were solubilized with 2% digitonin in 20 mM Tris-HCl (pH 7.4), 250 mM NaCl, 1 mM EDTA, and 10% glycerol. The mouse ortholog shares 93% identity (96% similarity) with Tim23. The 40 kDa protein interacts with Tim23 and Tim50. The 40 kDa protein contains a predicted transmembrane domain (residues 66–88) and a C-terminal CTD-like phosphatase domain (CPD, residues 146–274) similar to the catalytic domain of the RNA polymerase II CTD phosphatase (44–46). The same structural organization was found in the yeast Tim50 protein (29–31) and two other uncharacterized human proteins designated HSPC129 (GenBank™ accession no. AAH35744.1) and Dullard homolog (GenBank™ accession no. AAH9295.1) (Fig. 2, A and B). Alignment of the four protein sequences showed that only the primary sequence of the CPD is conserved (Fig. 2B).

The MTS of the 40 kDa protein was sufficient for targeting GFP to the mitochondria when expressed as a fusion protein with GFP (MTS-GFP) in MCF-7 cells (Fig. 3A, panels 2 and 4). MTS-GFP fusion protein exhibited a perinuclear punctate fluorescence, which colocalized with the specific mitochondrial stain Mitotracker Red (Fig. 3A, panel 6). Similar punctate fluorescence was observed with a full-length 40 kDa protein-GFP fusion protein (FL-GFP) (Fig. 3A, panel 1). Removal of the MTS and TM domain of the 40 kDa protein resulted in the expression of the truncated fusion protein (CTR-GFP) in the cytoplasm (Fig. 3A, panel 3). These observations indicate that the N terminus of the 40 kDa protein is indeed a MTS.

Interestingly, we observed that ~42% of the CTR-GFP-expressing cells showed signs of apoptosis such as rounding up and detachment from the plate, compared with ~22% in the case of the FL-GFP-expressing cells (Fig. 3B). However, only ~6–8% of the MTS-GFP- or GFP-expressing cells showed signs of apoptosis. Preincubation with z-VAD-fmk reduced apoptosis to almost background levels. These observations suggest that expression of the isolated CTR of the 40 kDa protein in the cytoplasm induces apoptosis by activating the caspase cascade.

The mitochondrial localization of the 40 kDa protein was also examined by immunofluorescence confocal microscopy and subcellular fractionation. Staining of HeLa cells with a 40 kDa protein-specific antibody and a second antibody against the mitochondrial Smac protein revealed similar superimposable punctate perinuclear pattern characteristic of mitochondrial localization (Fig. 4C). Subcellular fractionation of untreated or TNF- or TRAIL-treated HeLa cells showed that the majority of the 40 kDa protein resides in the mitochondrial fraction. Unlike cytochrome c, treatment of HeLa cells with TNF or TRAIL did not change the 40 kDa protein distribution (Fig. 3D). These results indicate that the 40 kDa protein is localized in the mitochondria and is not released by apoptotic stimuli. Examination of the subcellular localization of GFP-fusion constructs of HSPC129 and Dullard homolog revealed that these two proteins, which are homologous to yeast Tim50 and the 40 kDa protein, are also localized in the mitochondria (Fig. 3E, see also Fig. 5B).

The 40 kDa Protein Is Integrated in the Inner Mitochondrial Membrane—The 40 kDa protein contains a predicted transmembrane domain immediately after the MTS (Fig. 2A). This domain structure suggests that the 40 kDa protein could be integrated in the inner mitochondrial membrane after its import into the mitochondria. To address this question we prepared mitoplasts from purified 293T mitochondria by osmotic swelling followed by freeze-thawing. The intact mitochondria and mitoplasts were analyzed by immunoblotting with antibodies for the 40 kDa protein and Omi (a soluble intermembrane space protein). As expected, Omi was present in the intact mitochondria but not in the mitoplasts (Fig. 4A). However, the 40 kDa protein was detected both in the intact mitochondria and mitoplasts (Fig. 4A), indicating that it is likely anchored to the inner mitochondrial membrane via its N-terminal TM domain.

To further investigate the association of the 40 kDa protein...
with the mitochondria, we treated isolated mitochondria from human HeLa and 293T cells with increasing amounts of PK and monitored the accessibility of the 40 kDa protein by Western blotting with the 40 kDa polyclonal antibody that recognizes the C-terminal residues 293–306 (Fig. 4, B and C). Treatment of mitochondria with low concentrations of PK led to the complete degradation of the surface-exposed domain of Tom22, but not the soluble intermembrane space protein Omi, indicating that the mitochondrial preparations are intact. On the other hand, PK treatment resulted in limited degradation of the 40 kDa protein at low PK concentrations and increased with increasing PK concentrations until the bands disappeared at the highest PK concentrations (Fig. 4, B and C). These results indicate that the C-terminal CPD of the 40 kDa protein is exposed to the IMS, which became accessible to PK at high concentration as evidenced by the decrease in the intensity of the Omi band. Also consistent with these results, the IMS-exposed N-terminal domain of Tim23, which is recognized by the antibody used for Western blotting became accessible to PK as evidenced by the disappearance of the 15 kDa Tim23 initial degradation product at the highest PK concentrations. Tim23 was reported to have an unusual transmembrane topology, which spans both the outer and inner mitochondrial membranes (47). Its N-terminal domain (residues 1–50) is integrated into the outer membrane and exposes the N terminus to the cytosol, while the C-terminal half (residues 100–222) is integrated into the inner membrane, leaving residues 50–100 between the two domains exposed to the IMS. These results indicate that the N-terminal IMS domain of Tim23 runs parallel to the CPD of the 40 kDa protein within the IMS of the mitochondria.

To further assess the orientation of the 40 kDa protein in the mitochondria, we treated isolated mitochondria with trypsin and Western blotted the digested mitochondria with antibodies against the 40 kDa protein and Omi. Trypsin treatment resulted in complete degradation of Omi indicating that the IMS
and cytochrome empty pShag vector control were transfected into 293T cells as indicated was analyzed as above. Indicated concentrations of staurosporine for 5 h. Cytochrome transfected with the indicated siRNA for 48 h and then treated with the entry and plotted against duration of treatment (h).

**Fig. 7.** Potentiation of UV-induced cytochrome c release and apoptosis by siRNA-mediated depletion of human Tim50. A, the synthesized sense and antisense siRNAs used to suppress human Tim50 are shown below cDNA target sequence (TS). B, 293T cells were transfected with nonspecific-siRNA (control) and hTim50-siRNA for 48 h. The cells were then left untreated (0 h) or UV-irradiated for various times (h) as indicated. Cytochrome c release was analyzed by Western blotting as described under “Experimental Procedures.” Apoptosis was assessed by annexin V by measuring the percentage of cells with externalized phosphatidylserine. C, three independent experiments as in B were carried out and statistically evaluated. Ratios of released cytochrome c to unreleased COX were calculated by densitometry and plotted against duration of treatment (h). D, 293T cells were transfected with the indicated siRNA for 48 h and then treated with the indicated concentrations of staurosporine for 5 h. Cytochrome c release was analyzed as above. E, pShag-hTim50 siRNA expression vector or empty pShag vector control were transfected into 293T cell as indicated and cytochrome c release was analyzed as in B.

was accessible to trypsin (Fig. 4D, lower panel). Interestingly, the 40 kDa protein was only clipped into a 33-kDa band which was not further degraded (Fig. 4D, upper panel). From the size of this band and taking into consideration that the N terminus (residues 1–88) is inserted in the inner membrane and matrix, clipping must have occurred at residues C-terminal to the antibody epitope. To confirm this result, we expressed a C-terminal FLAG-tagged 40 kDa protein in 293T cells and then isolated mitochondria from these cells. Treatment of the isolated mitochondria with trypsin did not generate a 33 kDa FLAG antibody-detectable band indicating that trypsin clipping occurred at the C terminus of the 40 kDa protein, which resulted in the removal of the FLAG tag (Fig. 4D, middle panel). Taken together, these results demonstrate that the C-terminal CPD of the 40 kDa protein is exposed into the intermembrane space of the mitochondria.

**The 40 kDa Protein Interacts with Tim23—** The mitochondrial topology and close structural resemblance between the human 40 kDa protein and yeast Tim50 prompted us to examine whether the 40 kDa protein, like the yeast Tim50, interacts with Tim23. HeLa mitochondria were solubilized with 2% digitonin and subjected to immunoprecipitation with an antibody against human Tim23. The 40 kDa protein coprecipitated with anti-Tim23 monoclonal antibody but not with the control T7 monoclonal antibody (Fig. S5A) or human Tom22 antibody (data not shown). Moreover, ectopically expressed FLAG-tagged 40 kDa protein was also found to interact with endogenous Tim23 by co-immunoprecipitation assays (Fig. S5B). No interactions were detected between endogenous Tim23 and ectopically expressed FLAG-tagged HSPC129 or Dullard homolog (Fig. S5B). These observations suggest that the 40 kDa protein, but not HSPC129 or Dullard homolog, is most likely the human functional homolog of the yeast Tim50. Because of the obvious structural, sequence, and functional homology between the human 40 kDa protein and yeast Tim50, the 40 kDa protein was designated human Tim50 according to the established nomenclature for components of the mitochondrial import machinery (48).

**Human Tim50 Possesses Phosphatase Activity—** The presence of a CTD-like phosphatase domain at the C terminus of human Tim50 suggests that this protein might possess a phosphatase activity. To test this possibility we expressed N-terminal GST-tagged full-length or truncated human Tim50 in bacteria and purified the recombinant proteins on glutathione-Sepharose beads (Fig. 6A). The recombinant proteins were then tested for phosphatase activity using 10 mM pNPP as substrate, a standard substrate for a wide range of phosphatases (35). As shown in Fig. 6B, both the full-length human Tim50 and the isolated CTR (residues 110–353), but not the MTS (residues 1–50), catalyzed the conversion of pNPP to pNP and inorganic phosphate in a dose-dependent manner. However, the activity of the full-length protein was slightly higher than that of the isolated CTR (Fig. 6B). The human Tim50 phosphatase activity displayed a bell-shaped pH profile with an optimum activity around pH 4.0–6.0. Phosphatase activity declined sharply at basic pH (Fig. 6C).

Phosphatases are usually classified depending on their substrate specificity. Subsequently, two phosphotyrosine-containing peptides and one phosphothreonine-containing peptide were used to determine human Tim50 phosphatase specificity. As shown in Fig. 6D, incubation of human Tim50 and its isolated CTR with Tyr(P)-peptide substrate 1 (END(pY)INASL; derived from a highly conserved region of T cell PTP (49)) (DADE(pY)LIPQQG; corresponding to the autophosphorylation site of epidermal growth factor receptor (50)) (data not shown). Human Tim50 and its isolated CTR also exhibited activity toward Ser/Thr(P)-peptide RRA(pT)VA (Fig. 6E), even though their activity toward the Tyr(P)-peptide was almost ~2-fold greater than that toward the Ser/Thr(P)-peptide. Taken together, these data demonstrate that human Tim50 possesses a phosphatase activity toward both phospho-ser/tyr and -thre/yl residues and that its CTR is required for this activity.

**Identification of Human Tim50**

Human Tim50 was cloned using a cDNA library from HeLa cells. The isolated cDNA was sequenced and confirmed by comparison with published sequences. The protein sequence was used to design and test oligonucleotides for siRNA knockdown experiments. Human Tim50 was expressed in 293T cells and antibodies were generated using recombinant human Tim50 expressed in E. coli. The monoclonal antibody was used to co-immunoprecipitate human Tim50 from HeLa mitochondria. The antibody also coprecipitated the endogenous Tim23, indicating that human Tim50 interacts with Tim23.

The phosphatase activity of human Tim50 was measured using 10 mM pNPP as substrate. The enzyme displayed a bell-shaped pH profile with an optimum activity around pH 4.0–6.0. Phosphatase activity declined sharply at basic pH.

**Fig. 7.** Potentiation of UV-induced cytochrome c release and apoptosis by siRNA-mediated depletion of human Tim50. A, the synthesized sense and antisense siRNAs used to suppress human Tim50 are shown below cDNA target sequence (TS). B, 293T cells were transfected with nonspecific-siRNA (control) and hTim50-siRNA for 48 h. The cells were then left untreated (0 h) or UV-irradiated for various times (h) as indicated. Cytochrome c release was analyzed by Western blotting as described under “Experimental Procedures.” Apoptosis was assessed by annexin V by measuring the percentage of cells with externalized phosphatidylserine. C, three independent experiments as in B were carried out and statistically evaluated. Ratios of released cytochrome c to unreleased COX were calculated by densitometry and plotted against duration of treatment (h). D, 293T cells were transfected with the indicated siRNA for 48 h and then treated with the indicated concentrations of staurosporine for 5 h. Cytochrome c release was analyzed as above. E, pShag-hTim50 siRNA expression vector or empty pShag vector control were transfected into 293T cell as indicated and cytochrome c release was analyzed as in B.
Depletion of Human Tim50 by RNA Interference Accelerates Cytochrome c Release from the Mitochondria—Because mitochondria play a key role in apoptosis by releasing into the cytosol several regulators of apoptosis, we became interested in determining whether human Tim50 affects the mitochondrial response to apoptotic triggers. We decided to use small interfering RNA (siRNA) oligonucleotides to silence the expression of human Tim50 in cells and assess the effect of this treatment on UV- or staurosporine-induced cytochrome c release (Fig. 7A). Transfection of 293T cells with human Tim50-specific siRNA resulted in specific and efficient depletion of human Tim50 (Fig. 7B). UV irradiation caused more cytochrome c-release (~4-fold increase) and apoptosis in human Tim50-depleted cells compared with control cells (Fig. 7, B and C). Staurosporine stimulation also resulted in accelerated cytochrome c-release in human Tim50-depleted cells compared with control cells (Fig. 7D). The depletion of human Tim50 by itself resulted only in a small amount of cytochrome c release and apoptosis (Fig. 7B, 6th lane). Thus the depletion of human Tim50 appears to decrease the threshold for cytochrome c release from the mitochondria in response to apoptotic triggers. Additionally, to rule out any nonspecific effect of siRNA transfection on mitochondria, we took advantage of a recently developed DNA vector-based RNAi technique (38, 39) in which siRNAs are processed from a short RNA hairpin (hpRNA) transcribed under the control of the RNA polymerase III U6 promoter. Consistent with the above results, transfection of pShag-Tim50 in 293T cells also accelerated cytochrome c release from mitochondria (Fig. 7E). These results suggest that human Tim50 is critical for maintaining the integrity of the outer mitochondrial membrane.

Loss of Tim50 in Zebrafish Causes Massive Apoptosis in the CNS—To assess the importance of Tim50 in vivo, we utilized the zebrafish model system (51). A zebrafish ortholog of Tim50 (GenBank™ accession no. BC057522) was initially identified by BLAST searches of over 73,000 zebrafish EST sequences generated by the zebrafish Genome Resources Project. This cDNA encodes a 44 kDa protein of 387 amino acid residues with 64.5% identity (71.5% similarity) to human Tim50 (Fig. 8A). Furthermore, we placed Tim50 on both human and zebrafish
Fig. 9. Effects of zebrafish Tim50-morpholino antisense oligonucleotides on zebrafish development. A, morpholino antisense oligonucleotides (MO 1 and MO 2) can efficiently block zebrafish Tim50 translation in vitro. Increasing amounts of nonspecific, or MO 1 or MO 2 antisense oligonucleotides were incubated together with [35S]methionine containing reticulocyte lysates programmed with zebrafish Tim50 cDNA for 1 h. The translation products were fractionated by SDS-PAGE and detected by autoradiography. MO inputs are 0.25, 4, and 17 times the calculated mRNA amounts, respectively. B, the overall morphology of phenol red (WT) or zebrafish Tim50 MO (MO)-injected embryos at 48 hpf. Lateral views with anterior toward the left. C, heart morphology of phenol red (WT) or zebrafish Tim50 MO (MO)-injected embryos at 72 hpf. Arrows indicate dilated heart. D and E, Znp-1 staining of phenol red (WT) or zebrafish Tim50 MO (MO)-injected embryos at 28 hpf, respectively. Primary motorneuron axons project up in every segment. F and G, F-59 staining of phenol red (WT) or zebrafish Tim50 MO (MO)-injected embryos at 28 hpf, respectively. H–K, brain morphology of zebrafish embryos injected with phenol red (WT), zebrafish Tim50 mRNA (mRNA), zebrafish Tim50 MO (MO), or zebrafish Tim50 MO plus mRNA (MO + mRNA) at 24 hpf. L and M, acridine orange staining of phenol red (WT) or zebrafish Tim50 MO (MO)-injected embryos at 28 hpf. N, 30 embryos from control or zebrafish Tim50 MO-injected (MO) zebrafish embryos were collected and stained with acridine orange at 28 hpf. Acridine orange fluorescence of the head, trunk, and tail regions was divided into two categories, mild and severe. The percent of zebrafish embryos in each category was calculated.
we performed whole mount in situ hybridization using a labeled antisense RNA probe at different developmental stages. These data coupled with sequence alignments, confirm that the zebrafish gene is the ortholog of human Tim50.

To localize zebrafish tim50 transcripts during development, we performed whole mount in situ hybridization using a labeled antisense RNA probe at different developmental stages. At 48-hpf, zebrafish tim50 mRNA exhibited a high level of expression in the CNS, particularly in the brain tissues (Fig. 8C). This expression pattern was similar to that observed at 24 hpf and 72 hpf (data not shown) and consistent with that observed in human tissues (Fig. 1B).

In order to understand the functional importance of Tim50, we took a reverse genetic approach in zebrafish. We designed two morpholino antisense oligonucleotides that target the translation start codon of Tim50 (33, 34). Both MOs can efficiently inhibit in vitro translation of zebrafish Tim50 (Fig. 9A) and injection of either MO caused a similar phenotype. MO 2 was utilized for subsequent analysis since injection of MO 2 revealed a significant phenotype at lower doses. Microinjection of MO 2 (5 pmol/embryo at the 1–4 cell stage) consistently resulted in dysmorphic hearts, cardiac edema, and reduced motility with shortened body axes, whereas injection of phenol red produced no defects, demonstrating that these results were not injection artifacts (Fig. 9, B and C). At a range of 2.5–10 pmol/embryo, the resulting phenotypes were dose-dependent (Table I). Phenotypic abnormalities were scored in zebrafish Tim50-MO-injected embryos at 24 hpf and most of the zebrafish Tim50-MO-injected embryos died by 4 dpf. However, many of the defects could be partly rescued by co-injection of zebrafish Tim50 mRNA, the sequence of which was altered such that the zebrafish Tim50 MO could not bind without altering the primary amino acid sequence of the protein (Fig. 9, H–K, and Table II). These data indicate that the defects caused by the zebrafish Tim50 MO were specifically due to reduced levels of Tim50 protein.

To further investigate the function of the central nervous system and skeletal muscles, a simple touch response test was performed to assay locomotor behavior. We observed that Tim50 MO-injected embryos segregated into 3 different phenotypic groups: reduced motility, circling behavior, and spastic motility. To examine nervous system development in more detail, we stained embryos with Znp-1 antibody, to identify the axons of central primary motoneurons. In Tim50-MO-injected embryos these axons were found to frequently truncate prematurely (10–20%, Fig. 9, D and E). More significantly, myosin heavy chain staining with the F59 monoclonal antibody (52) showed that Tim50 MO injections results in disorganization and truncation of the skeletal muscle fibers (Fig. 9, F and G). Because proper organization and formation of muscle tissues depend on correct enervation, the observed muscle defects could be caused in part by the degeneration of the axons.

Increased apoptosis could be responsible for the neurodegenerative phenotype observed in the zebrafish Tim50-MO-injected embryos. Acridine orange is a vital dye that is widely used to label apoptotic cells during early embryonic development in zebrafish and other animals (53, 54). Acridine orange staining of Tim50 MO-injected embryos revealed increased numbers of apoptotic cells in the brain, spinal tube, and tail as compared with un.injected embryos (Fig. 9, L–N). These data indicate that loss-of-function of zebrafish Tim50 results in massive apoptosis in the CNS. The increase in cell death in the CNS is most likely due to loss of mitochondrial function and increased cytochrome c release.

### DISCUSSION

Human Tim50 was initially identified in a yeast two-hybrid screen for proteins that associate with the death domain of the TRAIL receptor DR5. Immunoprecipitation experiments subsequently showed that even though the C-terminal portion of human Tim50 could interact with the death domain of DR5, this interaction was not sufficient for the observed interactions between the endogenous proteins. Most notably, human Tim50 is present exclusively in the mitochondria, which explains the lack of interactions with endogenous TRAIL receptors that are located in the plasma membrane. Even upon induction of apoptosis by TRAIL stimulation, human Tim50 remains associated with the mitochondria. Recent studies suggest that yeast Tim50 facilitates import and sorting of presequence-containing mitochondrial precursor proteins most likely by direct interactions with positively charged matrix-targeting signals at their N termini (29–31). The death domain contains positively charged surfaces (55), which might explain the observed interactions between the isolated death domain and human Tim50 in the yeast two-hybrid and overexpression systems.

Human Tim50 is one of three human mitochondrial proteins identified in this study that have structural and sequence homology to the yeast Tim50. Human Tim50, together with Dullard homolog and HSPC129 contain a typical amphipathic

### TABLE I

| Survival | Head part | Cardiovascular defects | Body axes | Motility |
|----------|-----------|------------------------|-----------|----------|
| %        | %         | %                      | %         | %        |
| 60       | Smaller head | Dysmorphic heart         | Shorten axes | Reduced motility |
| 82       | Smaller eyes   | Reduced heart beating    | 91        | Curly tail |
| 86       | Smaller ears   | Decreased circulating blood | 84    | Spastic-like mutants |

* These phenotype frequencies (%) are representative of three experiments. Analysis was at 68–72 hpf.

### TABLE II

| Observed phenotypes | MO (n = 72) | MO + mRNA (n = 64) | mRNA (n = 62) | Uninjected (n = 60) |
|---------------------|------------|--------------------|---------------|---------------------|
| %                   | %          | %                  | %             | %                   |
| Survival            | 24 + 18/40 + 32 = 58 | 27 + 25/34 + 30 = 82 | 28 + 28/30 + 30 = 90 | 30 + 28/30 + 30 = 97 |
| Reduction of head   | 35 + 25/40 + 32 = 83 | 16 + 17/34 + 30 = 52 | 3 + 4/30 + 32 = 12 | 0                   |
| Cardiovascular defects | 33 + 31/40 + 32 = 89 | 21 + 22/34 + 30 = 67 | 3 + 2/30 + 32 = 8 | 0                   |
| Reduced motility    | 31 + 23/40 + 32 = 75 | 20 + 15/34 + 30 = 54 | 1 + 3/30 + 32 = 6 | 0                   |

The results are representative of two experiments. Analysis was at 68–72 hpf. Using the Mantel-Haenszel method to stratify (MO) and (MO + mRNA) by 2-by-2 Tables, p value for survival, reduction of head, cardiovascular defects, and reduced motility are 0.005, <0.001, 0.002, and 0.014, respectively.

Identification of Human Tim50

Human Tim50 was initially identified in a yeast two-hybrid screen for proteins that associate with the death domain of the TRAIL receptor DR5. Immunoprecipitation experiments subsequently showed that even though the C-terminal portion of human Tim50 could interact with the death domain of TRAIL receptors in an overexpression system, it failed to definitely show any interactions between the endogenous proteins. Most notably, human Tim50 is present exclusively in the mitochondria, which explains the lack of interactions with endogenous TRAIL receptors that are located in the plasma membrane. Even upon induction of apoptosis by TRAIL stimulation, human Tim50 remains associated with the mitochondria. Recent studies suggest that yeast Tim50 facilitates import and sorting of presequence-containing mitochondrial precursor proteins most likely by direct interactions with positively charged matrix-targeting signals at their N termini (29–31). The death domain contains positively charged surfaces (55), which might explain the observed interactions between the isolated death domain and human Tim50 in the yeast two-hybrid and overexpression systems.

Human Tim50 is one of three human mitochondrial proteins identified in this study that have structural and sequence homology to the yeast Tim50. Human Tim50, together with Dullard homolog and HSPC129 contain a typical amphipathic
mitochondrial targeting sequence at the N terminus followed by a transmembrane domain and a C-terminal CTD-like phosphatase domain. However, human Tim50, but not Dullard homolog or HSPC129, was found in a complex with Tim23 indicating that human Tim50 represents the functional homolog of the yeast Tim50. Human Tim50 also has a similar topology as the yeast Tim50 with its N terminus integrated in the inner membrane and its C-terminal CTD-like phosphatase domain exposed to the IMS. Nevertheless, few differences between human Tim50 and yeast Tim50 exist. Yeast Tim50 is made as a precursor protein that undergoes proteolytic processing to remove the N-terminal leader sequence, while no such protein was found that human Tim50 is processed at its N terminus (data not shown). The other main difference between the two proteins is that human Tim50 has a functional dual-specific phosphatase activity, while yeast Tim50 was reported not to have such an activity (30).

The regulation of cell death by reversible phosphorylation has been appreciated recently. The phosphorylation state of several mitochondrial proteins such as the antiapoptotic (Bcl-2, Bcl-XL) and proapoptotic (BAD, Bid, Bik) Bcl-2 proteins regulates their cellular activity and, therefore, cell survival and cell death (56). It is tempting to speculate that in addition to its translocase activity, human Tim50 might also function as a phosphatase for certain mitochondrial proteins such as the Bcl-2 family members to regulate the permeability of the outer mitochondrial membrane. Phosphorylation of Bcl-2 at serine 70 is required for Bcl-2 full and potent antiapoptotic function (57). A similar post-transcriptional modification was observed in other Bcl-2 family members, such as the antiapoptotic Bcl-xL protein and the proapoptotic Bcl-2 family members Bad, Bid, and Bik (58–62). Thus, dephosphorylation of these proteins by human Tim50 might affect their biological activity in the outer mitochondrial membrane.

In yeast the loss of Tim50 causes growth arrest and reduced viability indicating that this protein is essential for cell viability (29). Similarly the loss of Tim50 in cultured human cells appears to accelerate cytochrome c release and potentiates cell death in response to death stimuli. In addition, the loss of Tim50 in zebrafish embryos caused several developmental defects particularly in the CNS, which showed clear signs of neurodegeneration, most likely as a result of increased cell death. Tim50 helps in the transfer of precursor proteins from the Tom to the Tim23 complex and is required for the import of a large number of matrix proteins with classical presequences (63). Thus, when Tim50 is depleted from human cells or zebrafish embryos the mitochondria lose the ability to import a large number of matrix proteins, many of which are essential for energy production and maintenance of mitochondrial function. This could lead to the loss of mitochondrial integrity, permeabilization of the outer mitochondrial membrane, and the release of the mitochondrial apoptotic proteins that activate the caspase-dependent and -independent cell death pathways. Since neurons are fully differentiated cells that depend on mitochondria for survival, the loss of mitochondrial membrane integrity would undoubtedly cause increased neuronal cell death leading to neurodegeneration. In summary, our results indicate that Tim50 plays an essential role in maintenance of mitochondrial integrity and function not only in yeast but also in vertebrates. Loss of Tim50 function leads to mitochondrial dysfunction and cell death.

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