Identification of a Novel Mitochondrial Complex Containing Mitofusin 2 and Stomatin-like Protein 2*

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A reverse genetics approach was utilized to discover new proteins that interact with the mitochondrial fusion mediator mitofusin 2 (Mfn2) and that may participate in mitochondrial fusion. In particular, *in vivo* formaldehyde cross-linking of whole HeLa cells and immunoprecipitation with purified Mfn2 antibodies of SDS cell lysates were used to detect an ~42-kDa protein. This protein was identified by liquid chromatography and tandem mass spectrometry as stomatin-like protein 2 (Stoml2), previously described as a peripheral plasma membrane protein of unknown function associated with the cytoskeleton of erythrocytes (Wang, Y., and Morrow, J. S. (2000) *J. Biol. Chem.* 275, 8062–8071). Immunoblot analysis with anti-Stoml2 antibodies showed that Stoml2 could be immunoprecipitated specifically with Mfn2 antibody either from formaldehyde-cross-linked and SDS-lysed cells or from cells lysed with digitonin. Subsequent immunocytochemistry and cell fractionation experiments fully supported the conclusion that Stoml2 is indeed a mitochondrial protein. Furthermore, demonstration of mitochondrial membrane potential-dependent import of Stoml2 accompanied by proteolytic processing, together with the results of sublocalization experiments, suggested that Stoml2 is associated with the inner mitochondrial membrane and faces the intermembrane space. Notably, formaldehyde cross-linking revealed a "ladder" of high molecular weight protein species, indicating the presence of high molecular weight Stoml2-Mfn2 hetero-oligomers. Knockdown of Stoml2 by the short interfering RNA approach showed a reduction of the mitochondrial membrane potential, without, however, any obvious changes in mitochondrial morphology.

Mammalian mitofusins Mfn1 and Mfn2 are large GTPases of the mitochondrial outer membrane that mediate mitochondrial fusion (1–3). They also contain two coiled-coil domains or heptad repeats (HR1 and HR2)[2] (3). The major portion of each of the proteins, including the N terminus-proximal GTPase domain and HR1 and the C terminus-proximal HR2, is exposed to the cytosol (4). The attachment to the outer membrane is mediated by two membrane-spanning segments that are separated by a small intermembrane space loop and that are located between the HR1 and HR2 repeats. Human Mfn1 and Mfn2 are highly homologous proteins with 62% identity at the amino acid level with both, however, being essential proteins. The homozygous −/− Mfn1-null and −/− Mfn2-null mice die during embryonic development (5). The mouse embryonic fibroblasts (MEF), derived from the −/− Mfn1-null or −/− Mfn2-null embryos, have distinct mitochondrial morphology defects, and exhibit severe reduction in mitochondrial fusion activity (5). Thus, Mfn1 and Mfn2 may have different functions in mitochondrial fusion. Unlike MEF lacking Mfn1 or Mfn2, MEF lacking *both* mitofusins completely lack mitochondrial fusion capacity and show severe cellular dysfunction (6). Functions of Mfn2 that are independent of its fusion activity, *i.e.* controlling mitochondrial metabolism and repressing vascular smooth muscle cell proliferation, have also been reported (7, 8).

Mfn2 and Mfn1 have the capacity to form homo- and hetero-oligomers, as demonstrated by co-immunoprecipitation of tagged proteins (5, 9). At least one of the mitofusins is required on each of the adjacent mitochondria to promote mitochondrial fusion (10). The initial step of this process is characterized by tethering of two adjacent mitochondria via assembly of a mitofusin complex, which is mediated by HR2 forming a dimeric, antiparallel coiled-coil (10). The subsequent steps of mitochondrial fusion, including the exact function of the GTPase domains, are less well understood. In yeast, an interaction of the mitofusin homolog Fzo1, involving the GTPase domain and the HR domains, is essential for fusion (11). In the same organism, mitochondrial fusion has been reconstituted *in vitro*. Mitochondrial fusion proceeds through discreet and sequential mitochondrial outer and inner membrane fusion events, with both steps requiring GTP hydrolysis (12). A third GTPase, Opa1, appears also to be involved in the process of mitochondrial fusion (13). This GTPase is synthesized as a precursor, which is processed by a matrix-processing peptidase to a mature form, large Opa1 (l-Opa1). l-Opa1 is anchored to the inner mitochondrial membrane and can undergo another proteolytic processing, which cleaves off the transmembrane segment and forms the small form (s-Opa1) (14). In both yeast and mammals, the mitochondrial morphology is regulated through this proteolytic processing of Opa1 (Mgm1) in an ATP or mitochondrial membrane potential-dependent manner (14–17). Interestingly, Opa1-mediated fusion was reported to depend on Mfn1 but not on Mfn2 (18). The detailed differences in Mfn1 and Mfn2 function are, however, unclear at present. In yeast, an

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[2] The abbreviations used are: HR, heptad repeat; DMEM, Dulbecco's modified Eagle's medium; FA, formaldehyde; FACS, fluorescence-activated cell sorting; LC/MS/MS, liquid chromatography and tandem mass spectrometry; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; GSPBS, goat serum in PBS; siRNA, short interfering RNA; TMRE, tetramethylrhodamine ethyl ester perchlorate; IP, immunoprecipitation.

additional protein, Ugo1, which participates in mitochondrial fusion, has been identified (19, 20). This protein spans the outer mitochondrial membrane and interacts, via its N terminus-proximal cytosolic domain, with Fzo1 and, via its C terminus-proximal intermembrane space domain, with Mgm1, thus linking the outer and the inner membrane fusion machinery. However, neither an Ugo1 homolog nor additional proteins that facilitate mitochondrial fusion have been identified in mammals.

In this study, we have identified a novel complex containing Mfn2 and Stoml2. Our results suggest that Stoml2 is a novel mitochondrial intermembrane space/inner membrane-localized protein that forms a large hetero-oligomeric complex with Mfn2.

EXPERIMENTAL PROCEDURES

Plasmid Construction and in Vitro Transcription/Translation—To construct a plasmid encoding a His-tagged protein corresponding to amino acids 1–405 of Mfn2 (His-Mfn2-(1–405)), a 1215-nucleotide-long fragment of an MFN2 cDNA was amplified by PCR from pBSKIAA0214, which carries a human heart cDNA of MFN2 (21), and cloned into the NdeI-BamHI sites of pET-15b. The resulting pET-15b/Mfn2-(1–405) construct was verified by sequencing. To prepare an Mfn2 mammalian expression plasmid, a ~4550-nucleotide-long HindIII-NotI fragment of pBSKIAA0214 (21), carrying the entire MFN2 cDNA, was cloned into the HindIII and NotI sites of pcDNA3 (Invitrogen).

To prepare a Stoml2 expression plasmid, poly(A)$^+$ RNA isolated from HeLa cells was reverse-transcribed and PCR-amplified using the Superscript One-Step reverse transcriptase-PCR system (Invitrogen). The resulting 1107-nucleotide-long fragment containing the entire open reading frame of STOML2 was subcloned into pGEM-Easy T (Promega) and verified by sequencing. An EcoRI fragment containing the entire open reading frame of STOML2 was further cloned into pcDNA3, and a clone of pcDNA3/Stoml2 with the correct orientation with respect to the cytomegalovirus promoter was selected.

Cell Lines, Transfection, and siRNA Treatment—The HeLa human cell line (ATCC CCL-2) was grown in high glucose Dulbecco's modified Eagle's medium (DMEM; containing 4.5 g/liter glucose and 110 mg/liter sodium pyruvate, Invitrogen), supplemented with 10% fetal bovine serum. HeLa S3 (ATCC CCL-2.2), suspension-adapted HeLa cells, were grown as described (22). For transient expression experiments, cells were transfected with pcDNA3-derived plasmids using FuGENE 6 (Roche Applied Science) and incubated for ~36 h to allow transgene expression. For RNA interference-mediated knockdown experiments, cells were transfected with 20 nM siRNA directed against STOML2 mRNA or MFN2 mRNA (siGENOME SMART pool, Dharmacon) using Dharmafect I (Dharmacon), according the manufacturer's protocol, and incubated for 4 days to reduce Stoml2 or Mfn2 protein levels. In some experiments involving siRNA^{STOML2}, cells were also transfected for a second time, on the third day, and incubated for a total of 6 days.

Antibodies—His-Mfn2-(1–405) protein was expressed in the *Escherichia coli* strain BL21(DE3), and the recombinant protein was purified on nickel-nitrilotriacetic acid-agarose according to the manufacturer's protocol (Qiagen). The purified protein was used to generate antibodies in rabbits (Covance). The anti-Mfn2 antibodies were then blot-purified as described (23). The preparation and purification of anti-Mfn1 antibodies has been described (2). The following antibodies were also used: mouse anti-Bcl-2 monoclonal antibodies (Santa Cruz Biotechnology), goat anti-enolase polyclonal antibodies (Santa Cruz Biotechnology), rabbit anti-COXII serum (24), mouse anti-COXIV monoclonal antibodies (Molecular Probes), mouse anti-F$_1$-ATPase β-subunit monoclonal antibodies (Mitosciences), mouse anti-porin monoclonal antibodies (Calbiochem), rabbit anti-prohibitin polyclonal antibodies (Lab Vision), mouse anti-Opa1 monoclonal antibodies (BD Transduction Laboratories), chicken anti-Stoml2 (N-180) polyclonal IgY antibodies (Genway), chicken anti-Stoml2 (C-186) polyclonal IgY antibodies (Genway), mouse anti-α-tubulin monoclonal antibodies (Oncogene), and rabbit anti-actin polyclonal antibodies (Sigma).

Isolation and Purification of Mitochondria—Adherent cells were harvested from 20 T-175 flasks by trypsinization, washed once in PBS (140 mM NaCl, 3.8 mM NaH$_2$PO$_4$, 16.2 mM Na$_2$HPO$_4$) containing 10% calf serum and twice in NKM buffer (0.13 M NaCl, 5 mM KCl, 7.5 mM MgCl$_2$, 10 mM Tris-HCl, pH 7.4 (25 °C)). Cells in suspension culture, exponentially growing in a 3-liter volume, were harvested and washed twice in NKM buffer. The washed cells were resuspended in six volumes (relative to the packed cells) of TKM buffer (10 mM Tris-HCl, pH 6.7 (25 °C), 10 mM KCl, 0.15 mM MgCl$_2$) containing a Complete Mini protease inhibitor mixture (Roche Applied Science) and, after 2 min, were disrupted with a motor-driven Potter-Elvehjem glass-Teflon homogenizer until ~60–75% of the nuclei had been released (22). The remainder of the preparation of the mitochondria-enriched fraction was as described (22). The mitochondria were further purified by centrifugation through a discontinuous Percoll/metrizamide gradient as described (25, 26) but using an SW41 rotor. The purified mitochondria were washed and resuspended in STKM buffer (0.25 M sucrose in TKM) and stored at −80 °C.

Submitochondrial Localization and Import of Stoml2—For membrane association studies, purified mitochondria (1 mg/ml) were incubated in a buffer (0.05 mM EDTA, Complete Mini protease inhibitor mixture, EDTA-free (Roche Applied Science), 20 mM HEPES, pH 7.5 (25 °C)) for 15 min on ice. Then the same volume of distilled H$_2$O, 3.0 M NaCl, 0.2 M Na$_2$CO$_3$, or 2% Triton X-100 was added, and incubation was continued for 30 min. Samples were centrifuged at 100,000 × g_{av} for 30 min, and both pellet and supernatant fractions were analyzed by Western blotting.

For protease sensitivity studies, import-competent mitochondria (2 mg/ml) were incubated with 0.5 mg/ml proteinase K in a buffer (25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM KH$_2$PO$_4$, 0.05 mM EDTA, 5 mM MgCl$_2$, 10 mM Tris-HCl, pH 7.4 (25 °C)) in the presence of digitonin, as specified in Fig. 4, for 30 min on ice. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (5 mM final) and an equal volume of 2× Sample buffer (20% glycerol, 10% β-mercaptoetha-

nol, 4% SDS, 125 mM Tris-HCl, pH 6.8 (25 °C)). The samples were boiled for 5 min and analyzed by Western blotting.

Preparation of import-competent mitochondria and import of protein into isolated mitochondria were carried out as described (27). The Stoml2 was synthesized in the rabbit reticulocyte lysate-based TNT T7-coupled transcription/translation system (Promega), in the presence of [^{35}S]methionine (Amersham Biosciences), according the manufacturer's protocol.

[^{35}S]Met Labeling, Formaldehyde (FA) Cross-linking, and Immunoprecipitation—To label cells with [^{35}S]methionine, 1.6×10^6 cells were grown in a 55-cm^2 plate containing 4 ml of low methionine (2×10^{-2} mM methionine)-DMEM supplemented with 10% dialyzed fetal bovine serum in the presence of 0.5 mCi Expre^{35}S^{35}S protein labeling mixture (1000 Ci/mmol) for 16 h. After the labeling period, the cells were washed twice in regular culture medium and processed for FA cross-linking.

^{35}S-Labeled or unlabeled cells were scraped with a rubber policeman and resuspended in culture medium ($\sim 2 \times 10^6$/10 ml). The cell suspension was mixed with a one-tenth volume of 11% FA (Polysciences) in PBS and incubated under mixing for 1–15 min at room temperature. Then, an equal volume of 250 mM glycine in PBS was added, and incubation was continued for 15 min to quench the unreacted FA. The cells were recovered by centrifugation and washed sequentially in PBS containing 1% bovine serum albumin and PBS.

For denaturing immunoprecipitation, $\sim 1 \times 10^6$ cells were lysed in 0.05 ml of buffer A (1% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 7.5 (25 °C)) with vortexing in the presence of acid-washed glass beads. Then 0.85 ml of Tween-IP buffer (0.5% Tween 20, 150 mM NaCl, 0.1 mM EDTA, 50 mM Tris-HCl, pH 7.5 (25 °C)) containing the Complete Mini protease inhibitor mixture was added, and insoluble debris were removed by centrifugation at $16,000 \times g_{av}$ for 10 min. The supernatants (same amounts of total proteins) were incubated with the appropriate purified rabbit antibodies for 3 h at 4 °C. Then, 0.05 ml of the protein A-Sepharose suspension (100 mg of protein A-Sepharose (Sigma)/2.8 ml of a buffer (1 mg/ml bovine serum albumin, 1 mM NaN$_3$, 10 mM Tris-HCl, pH 7.5)) was added, and the incubation was continued for 30 min. The immune complexes captured on Sepharose beads were centrifuged through a sucrose cushion (3.42 g of sucrose/10 ml of Tween-IP buffer), washed twice with Tween-IP buffer and once with 1% 2-mercaptoethanol, and solubilized in Sample buffer (10% glycerol, 5% β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8 (25 °C)). To reverse the cross-linking, the resulting samples were heated to 95 °C for 25 min. The proteins were separated by SDS-PAGE and detected by autoradiography or, after the transfer to a membrane, by immunoblotting as described below.

In the case of the large scale experiment (Fig. 2*B*), mitochondria, prepared by differential centrifugation from 21 g of HeLa S3 cells, were treated with 1% FA in buffer B (250 mM Sucrose, 10 mM KCl, 0.15 mM MgCl$_2$, 20 mM HEPES, pH 7.2 (25 °C)) for 15 min. The FA was quenched by the addition of an equal volume of 250 mM glycine in buffer B. The mitochondrial pellet was then lysed in 15 ml of buffer A, insoluble debris were removed by centrifugation, and the resulting supernatant was diluted 18-times with Tween-IP buffer. Protein complexes were captured using purified anti-Mfn2 antibodies covalently attached to Dynabeads-protein A (Dynal) with dimethyl pimelimidate dihydrochloride, according to the manufacturer's protocol, and were eluted with a buffer (500 mM NaCl, 100 mM glycine, pH 2.8 (25 °C)). The eluate was neutralized and concentrated on an Amicon Ultra centrifugal device (Millipore). Proteins were heat-treated, separated by SDS-PAGE, and detected by silver staining, and selected protein bands were excised. The further sample preparation and analysis was done by the Protein/Peptide Microanalytical Laboratory at the California Institute of Technology. Briefly, gel slices were treated with sequencing grade trypsin, and the resulting peptides were analyzed by liquid chromatography and tandem mass spectrometry (LC/MS/MS). The identified peptide sequences were matched to the National Center for Biotechnology Information sequence data base using MASCOT software. The probabilities-based Mowse scores and the coverage of the proteins with the identified peptides were as follows (probability Mowse score/sequence coverage in percent): Stoml2, 42A (430/26), Stoml2, 42B (430/26), Mfn1 (500/28), Mfn2 (570/33).

For nondenaturing immunoprecipitation, $\sim 1 \times 10^6$ cells were lysed in 0.8 ml of a co-IP buffer (0.5% digitonin, 120 mM NaCl, Complete Mini protease inhibitor mixture, 50 mM HEPES, pH 7.5 (25 °C)) for 15 min at 4 °C. The insoluble debris were removed by centrifugation at $16,000 \times g_{av}$ for 10 min. The resulting supernatants were incubated with the appropriate antibodies and protein A-Sepharose as described above. The immune complexes captured on Sepharose beads were centrifuged through a sucrose cushion (3.42 g of sucrose/10 ml of co-IP buffer), washed three times with co-IP buffer and once with co-IP buffer lacking digitonin, and further analyzed by Western blotting.

Sample Preparation and Western Blot Analysis—To remove contaminating rabbit IgGs present in the rabbit reticulocyte lysate-based TNT T7-coupled transcription/translation system, which could interfere, in the Western blot analysis, with detection of *in vitro* synthesized Stoml2, samples of *in vitro* synthesized Stoml2 were incubated with protein A-Sepharose beads in an appropriate buffer (50 mM NaCl, 0.1 mM EDTA, 0.1% Tween 20, 25 mM Tris-HCl, pH 7.5 (25 °C)) for 1 h at 4 °C, and the Sepharose beads were then removed by centrifugation.

The FA-cross-linked cells or FA-cross-linked purified mitochondria were incubated with buffer containing 2% SDS, 100 mM NaCl, and 10 mM Tris-HCl, pH 8.0 (25 °C) for 15 min and then centrifuged to remove insoluble debris, and the resulting extracts were used for Western blot analysis.

The total protein concentration in samples of whole cells, cell extracts, or purified mitochondria was determined by the Bradford method or by the bicinchoninic acid method (28, 29). Samples (whole cells, subcellular fractions, purified mitochondria, protein A-Sepharose-treated *in vitro* synthesized proteins, or immunoprecipitated proteins) were mixed with an equal volume of 2× Sample buffer and analyzed by SDS-PAGE. Rainbow (Amersham Biosciences RPN756), Magic Mark XP (Invitrogen), or Kaleidoscope (Bio-Rad) size markers were analyzed in parallel. Proteins were then electrophoretically transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences) in a previously described transfer buffer (30) modified to contain 0.037% SDS. Blocking, first and second

antibody incubations, and washes were carried out in PBSTw (PBS plus 0.1% Tween 20) or PBSTw containing 5% nonfat milk. The specific protein complexes were identified by autoradiography using the Super-Signal West Pico chemiluminescence reagent (Pierce).

Fluorescence Microscopy and FACS Analysis—Cells were grown in a chambered coverglass (Lab-Tek), and the medium was replaced ~12 h before an experiment. Cells were stained with 25 nM tetramethylrhodamine ethyl ester perchlorate (TMRE, Molecular Probes) or 50 nM Mitotracker Red CMXRos (Molecular Probes) in DMEM without serum for 30 min and washed again with culture medium. Microscopy was performed using an inverted Nikon Diaphot microscope equipped with a Nikon G2A filter cube, a Zeiss Planapo 63×/1.4 oil objective, and a Nikon D50 digital camera. For FACS analysis, cells were stained with 10 nM TMRE, trypsin-harvested, resuspended in PBS containing 10 nM TMRE, and analyzed by a FACSCalibur analyzer (Bio-Rad). Oxygen consumption measurements were done as described previously (31).

Confocal Immunofluorescence Microscopy—Cells grown on glass coverslips were sequentially incubated in 2% FA in PBS, PBS, anhydrous methanol, PBS, and 2% goat serum in PBS (GSPBS) containing 0.5% Triton X-100. The coverslips were then incubated with anti-Stoml2 antibodies (C-186; diluted 1:50 in GSPBS) and anti-COXIV antibodies (diluted to 5 μg/ml in GSPBS) for 3 h at 37 °C in a humidified chamber. As a negative control, nonimmune chicken IgY antibodies (Aves Labs) were used instead of the anti-Stoml2 antibodies. After four washes in GSPBS, the coverslips were incubated with 1:50-diluted fluorescein isothiocyanate-conjugated donkey anti-chicken IgY and 1:50-diluted rhodamine Red-X-conjugated goat anti-mouse IgG (both from Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After four washes in PBS, the coverslips were mounted onto microscope slides in FluoroGuard antifade reagent (Bio-Rad) and analyzed on a Zeiss 410 laser-scanning microscope equipped with a 488-nm argon and a 543-nm helium neon laser, a Zeiss 63×/1.25 oil objective, and Zeiss LSM software.

RESULTS

We used *in vivo* FA cross-linking of HeLa cells to preserve Mfn2 protein complexes and to identify possible Mfn2-interacting proteins. FA is a highly specific cross-linker that is reactive with primary amines within 2 Å of one another, is easily reversible, and is used to preserve protein-protein, protein-DNA, or protein-RNA complexes (32). To detect Mfn2, immunoblotting with antibodies generated against a large portion of the Mfn2 protein was used. These antibodies detected a major immunoreactive protein in the untreated cell lysates (Fig. 1*A*, *left panel, first lane*), identified as Mfn2, as it was specifically increased in amount when HeLa cells were transfected with an Mfn2 expression vector (see Fig. 7*B*) and was specifically reduced when HeLa cells were treated with siRNA[MFN2] (see Fig. 7*C*). The minor, slower migrating protein in the untreated cell lysates (Fig. 1*A*, *left panel, first lane*) was identified as Mfn1, as it was specifically reduced in amount when HeLa cells were treated with siRNA[MFN1] (see Fig. 7*C*). When HeLa cells were treated with 1% FA for 15 min, high molecular weight FA-cross-
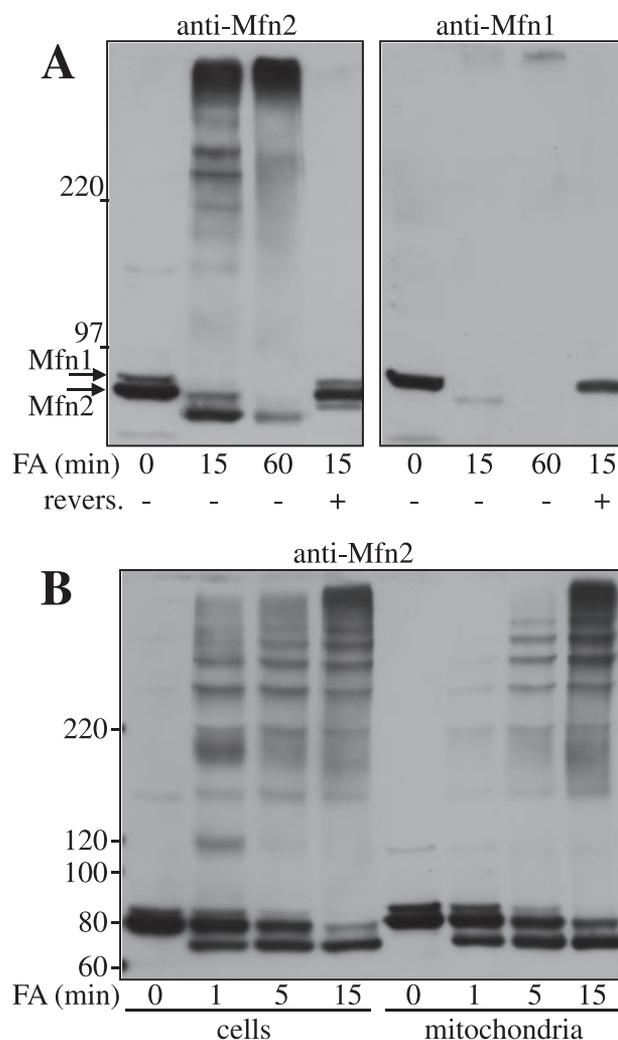

FIGURE 1. **Formaldehyde cross-linking reveals Mfn2 oligomerization.** *A*, HeLa CCL2 cells were treated for 0, 15, or 60 min with FA, and SDS cell lysates were analyzed by Western blotting with anti-Mfn2 or anti-Mfn1 antibodies. Some samples of SDS lysates were heated to reverse the cross-linking (*revers.*) as indicated. *B*, HeLa CCL2 cells or purified mitochondria obtained from HeLa S3 cells were treated for 0, 1, 5, or 15 min with FA, and their SDS lysates were analyzed by Western blotting with anti-Mfn2 antibodies. High exposures are shown for the purpose of visualization of the high molecular weight cross-linked bands. Under these conditions the intensities of the monomeric bands are underrepresented.

linked products were detected by immunoblotting with anti-Mfn2 antibodies (Fig. 1*A*, *left panel*). Longer FA treatments led to a loss of the distinct high molecular weight bands immunoreactive with anti-Mfn2 antibodies (Fig. 1*A*, *left panel*). Because anti-Mfn2 antibodies weakly cross-react with Mfn1, it was possible that at least some of the anti-Mfn2 immunoreactive cross-linked products were due to the presence of Mfn1. Furthermore, Mfn2 has been shown to interact with Mfn1 (5, 9). However, almost no high molecular weight cross-linked products were detected on immunoblots treated with anti-Mfn1 antibodies (Fig. 1*A*, *right panel*). This indicates that the ladder-like bands consist specifically of Mfn2 homo- or hetero-oligomers and that most of the cross-linked Mfn1 products were not soluble or were not electrotransferred. In addition, a single, faster migrating, cross-linked product was detected with anti-Mfn2 or anti-Mfn1 antibodies, suggesting the presence of

intramolecularly cross-linked Mfn2 and Mfn1 products (Fig. 1). Five minutes of FA cross-linking of whole cells led to an optimal resolution of the ladder-like pattern detected with anti-Mfn2 antibodies while minimizing products barely entering the gel (Fig. 1*B*). For a number of reasons, including the unequal efficiencies of transfer of small *versus* large proteins, the signals on a Western blot do not give an accurate representation of the amount of target protein. Nevertheless, to obtain an approximation of the levels of Mfn2 in the various bands on the blots, we quantified the chemiluminescent signals of the blots on film exposures lower than those shown in Fig. 1*B*. Thus, after 5 min of FA treatment, and relative to the amount of Mfn2 present in the untreated sample, ~12% of the total Mfn2 was estimated to be present as the uncross-linked Mfn2 monomer, ~16% as the internally cross-linked Mfn2, and ~8% as the discrete high molecular weight cross-linked products. Some of the remaining unaccounted for (~64%) Mfn2 protein was present in high molecular weight heterogeneous material. The rest was not soluble or was not electrotransferred. Short times of cross-linking (1–15 min of FA treatment) resulted in a relatively constant amount of Mfn2 (~6–9%) present in discrete high molecular weight cross-linked products. Importantly, a reversion of the cross-linking resulted in conversion of most of the soluble cross-linked species back to Mfn2 and Mfn1 monomers (Fig. 1*A*). Most of the high molecular weight Mfn2-immunoreactive products were detected not only by FA cross-linking of whole cells but also by FA cross-linking *in organello*, that is by cross-linking of highly purified mitochondria, indicating that the putative Mfn2 complexes are preserved in isolated mitochondria (Fig. 1*B*).

Identification of Stomatin-like Protein 2 (Stoml2)—To analyze the composition of the formaldehyde-cross-linked oligomers, SDS lysates of [^{35}S]Met-labeled and FA-cross-linked HeLa cells were immunoprecipitated with anti-Mfn2 antibodies, and the cross-linking was reversed by heating the immunoprecipitates to 95 °C to release individual proteins (Fig. 2*A*). Several proteins appeared to be specifically precipitated with anti-Mfn2 antibodies only when cells had been treated with FA. Among them, a ~42-kDa protein was detected (Fig. 2*A*). The ~42-kDa protein was not detected in FA-cross-linked cells immunoprecipitated with preimmune serum or anti-Mfn1 antibodies (data not shown). Previous experiments indicated that a similar pattern of the Mfn2-immunoreactive complexes is observed if purified mitochondria are used for the cross-linking (Fig. 1*B*). Indeed, the ~42-kDa protein was also detected by FA cross-linking *in organello* and immunoprecipitation with anti-Mfn2 antibodies (data not shown). Large scale preparation of the Mfn2 immunoprecipitate of FA-cross-linked mitochondrial fraction yielded two dominant protein bands, 42A and 42B, as well as ~74- and ~70-kDa proteins, detected by silver staining (Fig. 2*B*). LC/MS/MS analysis of trypsin digests identified both the 42A and 42B bands as Stoml2. Most likely, the 42B form of Stoml2 was generated by a nonspecific proteolysis during the large scale purification, because only a singleton band was detected by immunoblotting with anti-Stoml2 antibodies of whole cell lysates (see Fig. 5*A*). The LC/MS/MS analysis also confirmed that the 74-kDa protein is Mfn1 and the 70-kDa protein is Mfn2. The relatively high
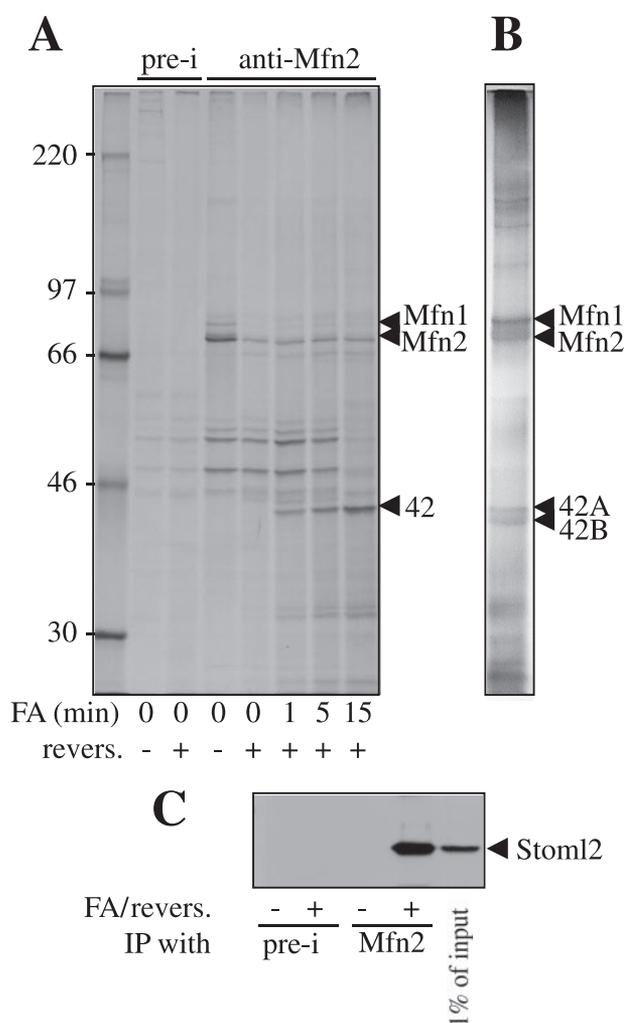
FIGURE 2. **Stoml2 is specifically immunoprecipitated with Mfn2 antibodies from FA-cross-linked cells.** *A*, [^{35}S]Met-labeled HeLa CCL2 cells were treated with FA, lysed, immunoprecipitated with either preimmune serum (*pre-i*) or anti-Mfn2 antibodies, heat-treated to 95 °C to reverse cross-linked products (indicated as *revers.*), and analyzed by SDS-PAGE and autoradiography. Time (in minutes) of FA treatment is indicated. The nonspecific ~47- and ~52-kDa bands were absent in the HeLa S3 immunoprecipitates (not shown). *B*, the mitochondrial fraction, prepared by differential centrifugation from HeLa S3 cells, was treated with FA for 15 min, lysed with SDS, and immunoprecipitated with anti-Mfn2 antibodies attached to magnetic beads. The resulting immunoprecipitate was heat-treated and analyzed by SDS-PAGE and silver staining. *C*, HeLa CCL2 cells were treated as indicated with FA for 15 min, lysed with SDS, immunoprecipitated with either preimmune serum or anti-Mfn2 antibodies, heat-treated, and analyzed by Western blotting with Stoml2 antibodies. In parallel, 1% of total cell lysate used for immunoprecipitation (*1% of input*) was analyzed.

amount of Mfn1 in the immunoprecipitate, when compared with Mfn2, is likely because of contributions of the immunoprecipitated Mfn1-Mfn2 cross-linked products as well as the cross-reactivity of the anti-Mfn2 antibodies with Mfn1. Importantly, anti-Stoml2 antibodies detected Stoml2 in the anti-Mfn2 immunoprecipitate prepared from FA-cross-linked cells but failed to detect the Stoml2 in the anti-Mfn2 immunoprecipitate prepared from cells without cross-linking (Fig. 2*C*). About 10% of total cellular Stoml2 was precipitated with anti-Mfn2 antibodies in this experiment. Thus, we confirmed the presence of Stoml2 in Mfn2-containing FA-cross-linked products, suggesting the presence of Mfn2-Stoml2 hetero-oligomers.
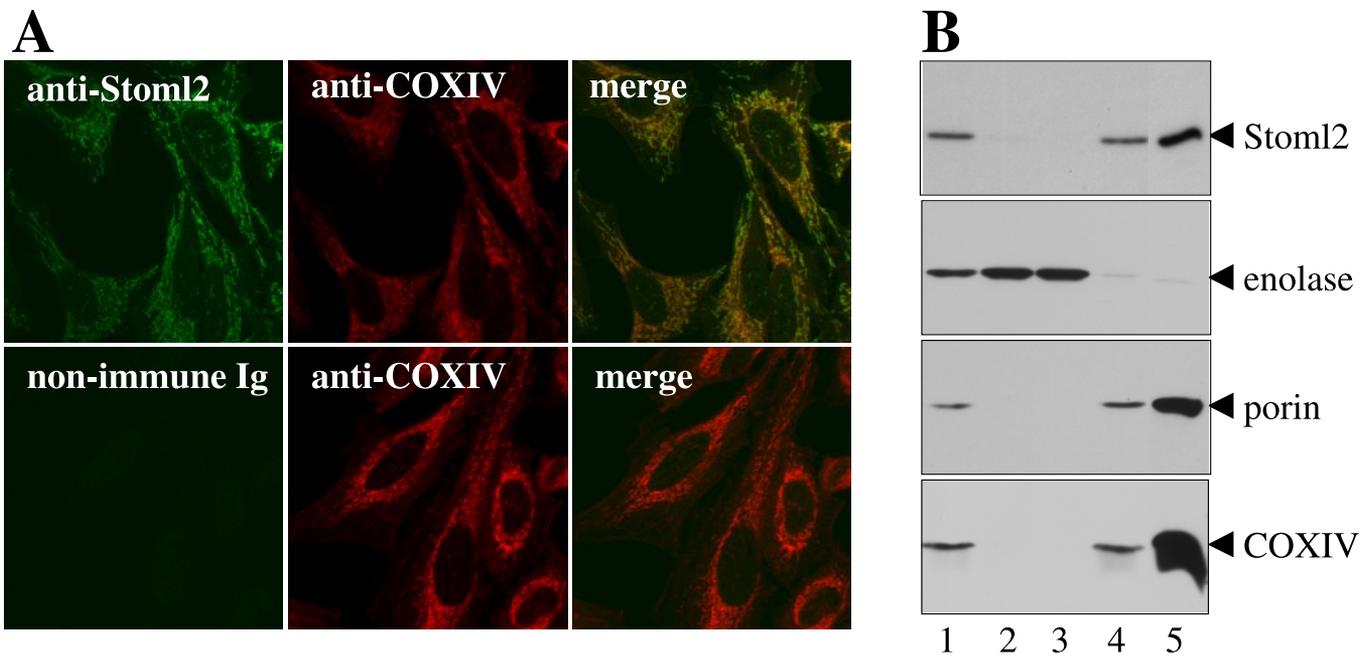
FIGURE 3. **Stoml2 is a mitochondrial protein.** *A*, HeLa CCL2 cells were fixed, processed for indirect immunocytochemistry, and analyzed by confocal fluorescence microscopy. Representative images of cells stained with anti-Stoml2 antibodies (or nonimmune Ig, in *green*) or anti-COXIV antibodies (in *red*) and merged images are shown. *B*, HeLa CCL2 cells were fractionated, and the resulting mitochondrial fraction was further purified on a Percoll/metrizamide gradient. Selected fractions (10 μg of protein) were analyzed by Western blotting with anti-Stoml2, anti-enolase, anti-porin, or anti-COXIV antibodies. *Lane 1*, whole cell homogenate; *lane 2*, 1300 × *g* supernatant; *lane 3*, 6700 × *g* supernatant; *lane 4*, 6700 × *g* pellet; *lane 5*, Percoll/metrizamide-purified mitochondria.

Mitochondrial Localization of Stoml2—Stoml2 had previously been reported to be a plasma membrane-associated protein of unknown function (33). However, several proteomics studies have detected Stoml2 in purified mitochondria, and, importantly, in one study, hemagglutinin-tagged Stoml2 was found to co-localize with a mitochondrial marker (34–37). To confirm the mitochondrial localization, we carried out an indirect immunocytochemistry experiment using anti-Stoml2 antibodies. Images obtained by confocal fluorescence microscopy revealed that the endogenous Stoml2 co-localizes with a mitochondrial marker, COXIV, in HeLa cells (Fig. 3*A*). In addition, cell fractionation followed by immunoblotting revealed that Stoml2 co-fractionates with the mitochondrial markers porin and COXIV. Furthermore, it was highly enriched in purified mitochondria, which appeared to be free of a cytosolic marker, enolase (Fig. 3*B*).
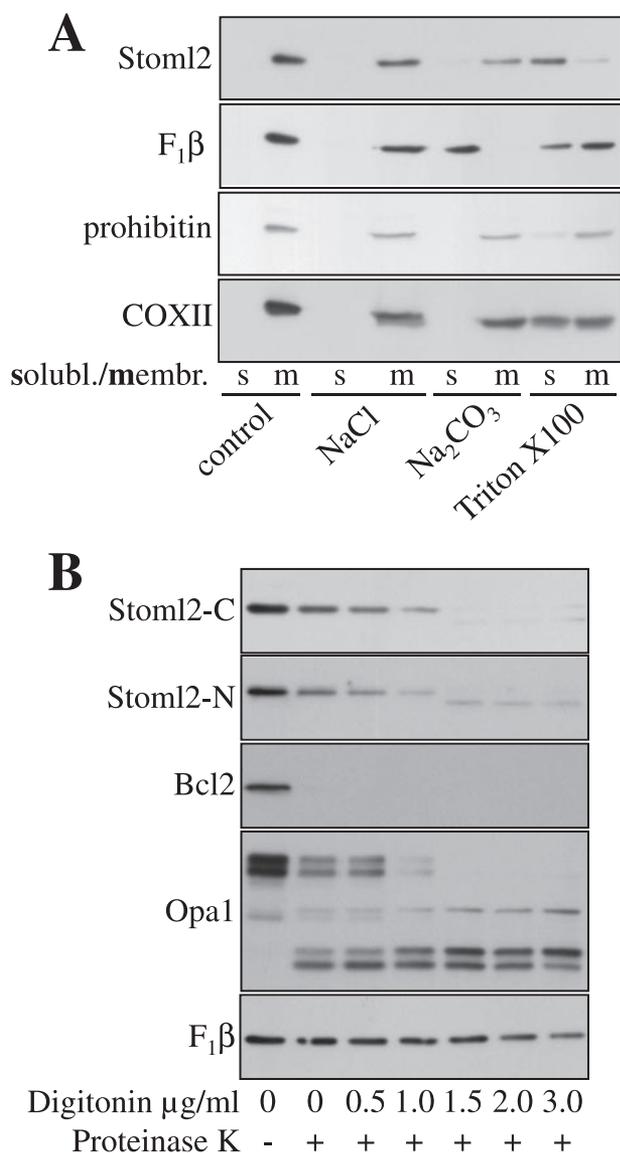
Submitochondrial Localization of Stoml2—To determine whether Stoml2 is a membrane-bound or soluble mitochondrial protein, purified mitochondria were treated with sodium chloride, sodium carbonate, or Triton X-100, and the resulting washes and mitochondrial membrane-containing pellets were analyzed by immunoblotting (Fig. 4*A*). Stoml2 remained associated with the membrane fraction after sodium chloride treatment, which removes soluble mitochondrial proteins, and even after the sodium carbonate treatment, which stripped off the F_1-ATPase β-subunit, a peripheral membrane protein (38). Stoml2 was nearly completely solubilized from the mitochondrial membranes by 1% Triton X-100 treatment, which only partially solubilized the F_1-ATPase β-subunit and the integral membrane protein COXII, while failing to solubilize another integral membrane protein, prohibitin. Because Stoml2 has no predicted transmembrane domain, its integral membrane pro-

tein-like behavior may be due to a post-translational modification, such as a fatty acylation or glycosylphosphatidylinositol modification, which mediates the membrane anchoring.

To dissect the Stoml2 submitochondrial localization further, purified mitochondria were treated with increasing concentrations of digitonin in the presence of proteinase K, and the resulting fractions were analyzed by immunoblotting (Fig. 4*B*). Bcl2, an outer mitochondrial membrane protein that faces the cytosol, was sensitive to proteinase K even in the absence of digitonin. Full-length Stoml2, detected with antibodies against the C-terminal half of the protein (Stoml2-C) or with antibodies against the N-terminal half of the protein (Stoml2-N), was sensitive to proteinase K digestion at about the same digitonin concentrations needed to digest the intermembrane space protein Opa1. Under these conditions, the mitochondrial matrix marker F_1-ATPase β-subunit was largely resistant to proteinase K digestion. Proteinase K treatment under higher digitonin concentrations led to the generation of a proteolytic fragment of Stoml2 that could be detected by either anti-Stoml2-C or anti-Stoml2-N antibodies. The detection of this fragment suggests that a part of Stoml2 is inaccessible to the protease, perhaps because of a tight membrane association. Overall, these experiments indicated that Stoml2 is at least partially exposed to the mitochondrial intermembrane space compartment. The partial sensitivity of Opa1 and Stoml2 to proteinase K in the absence of digitonin is likely due to the presence in the mitochondrial preparation of some mitochondria with broken outer membranes, and therefore this result did not affect the interpretation of the experiments.
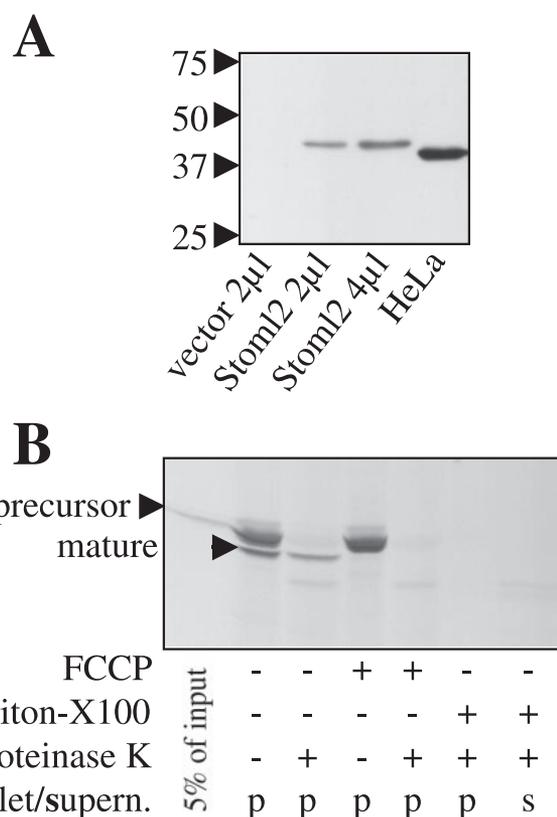
In the course of analysis of Stoml2, we noticed that Stoml2 synthesized *in vitro* from human cDNA migrates more slowly than the endogenous Stoml2 of HeLa cells (Fig. 5*A*). This phe-

FIGURE 4. **Mitochondrial sublocalization of Stoml2.** *A*, membrane association of Stoml2. Purified mitochondria prepared from HeLa S3 cells were disrupted by hypotonic shock (*control*) and treated with 1.5 M NaCl, 0.1 M Na_2CO_3 (pH 11.5), or 1% Triton X-100. After the treatment, mitochondrial membranes (*m*) were separated from soluble material (*s*), as detailed under "Experimental Procedures," and both fractions were analyzed by Western blotting with anti-Stoml2, anti-F_1-ATPase β-subunit, anti-prohibitin, or anti-COXII antibodies. *B*, proteinase K sensitivity of Stoml2. Purified mitochondria were left untreated, treated with proteinase K, or treated with proteinase K in the presence of increasing concentrations of digitonin and analyzed by Western blotting with anti-Stoml2-C, anti-Stoml2-N, anti-Bcl-2, anti-Opa1, or anti-F_1-ATPase β-subunit antibodies.

FIGURE 5. **Stoml2 import into isolated mitochondria is dependent on mitochondrial membrane potential and is accompanied by proteolytic processing.** *A*, electrophoretic mobility of Stoml2. *In vitro* synthesized [^{35}S]Met-labeled Stoml2 (2 or 4 μl) from a vector carrying STOML2 cDNA, the products of a control reaction carried out in the presence of an empty vector, as well as 10 μg of HeLa CCL2 cell lysate were analyzed by Western blotting with anti-Stoml2 antibodies. *B*, Stoml2 import. *In vitro* synthesized [^{35}S]Met-labeled Stoml2 was incubated with isolated mitochondria and in some samples, as indicated, in the presence also of carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (*FCCP*) as detailed under "Experimental Procedures." Following import, samples were left untreated, treated with proteinase K, or treated with proteinase K and 1% Triton X-100. After centrifugation, the resulting pellet (*p*) and supernatant (*s*) fractions were analyzed by SDS-PAGE and autoradiography. In parallel, 5% of the *in vitro* synthesized Stoml2, used for the import reaction, was analyzed (5% *of input*). The *in vitro* synthesized Stoml2 (*precursor*) and the imported and processed form of Stoml2 (*mature*) are each indicated by an *arrowhead*.
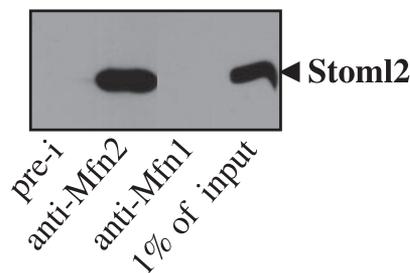
FIGURE 6. **Mfn2 and Stoml2 form a complex.** HeLa CCL2 cells were lysed with digitonin and immunoprecipitated with preimmune serum (*pre-i*) or anti-Mfn2 or anti-Mfn1 antibodies. The resulting immunoprecipitates and 1% of total cell lysate used for immunoprecipitation (1% *of input*) were analyzed by Western blotting with anti-Stoml2 antibodies.

nomenon is often an indication of a proteolytic processing that accompanies the import of some proteins into the mitochondrial matrix or the inner membrane or the intermembrane space. Therefore, we carried out an import experiment using Stoml2 synthesized *in vitro* and import-competent mitochondria prepared from HeLa cells (Fig. 5*B*). Compared with the *in vitro* synthesized (precursor) form of Stoml2, a faster migrating (mature) form of Stoml2 was detected upon incubation of the precursor with mitochondria. This mature form of Stoml2 was resistant to subsequent proteinase K treatment of intact mitochondria but was sensitive to proteinase K treatment of Triton
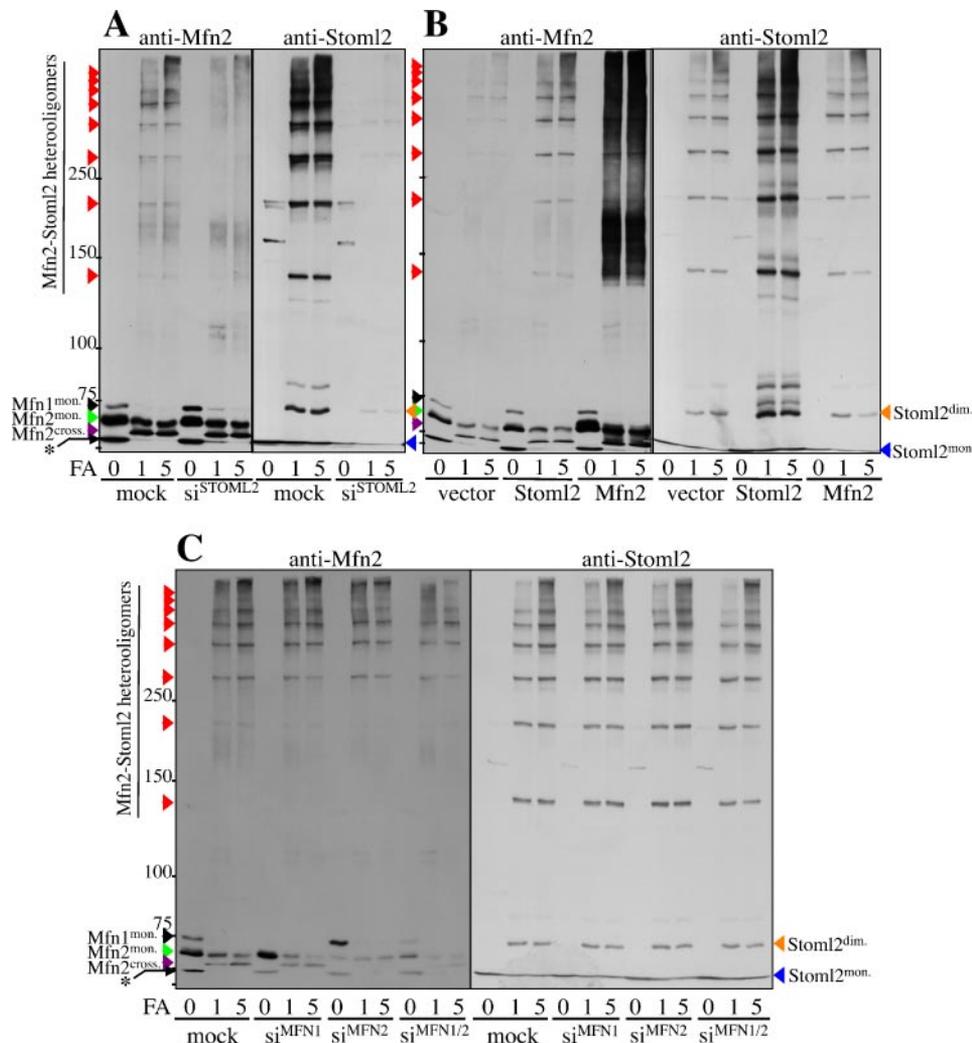
X-100-solubilized mitochondria. In addition, the mature form of Stoml2 was not detected if the import reaction was carried out in the presence of the ionophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone, which dissipates the mitochondrial membrane potential across the inner mitochondrial

FIGURE 7. **Stoml2 and Mfn2 form large complexes captured by FA cross-linking.** *A*, mock-transfected HeLa CCL2 cells (*i.e.* cells transfected with the lipid carrier) or cells transfected with siRNA^{STOML2} were treated with FA for the indicated periods of time (in minutes), lysed with SDS, and analyzed by Western blotting with anti-Mfn2 antibodies followed by reblotting with anti-Stoml2 antibodies. *B*, cells transfected with an empty vector, a vector carrying STOML2 cDNA, or a vector carrying MFN2 cDNA were treated and analyzed as described in *A*. *C*, mock-transfected HeLa CCL2 cells or cells transfected with siRNA^{MFN1}, siRNA^{MFN2}, or siRNA^{MFN1}/siRNA^{MFN2} were treated and analyzed as described in *A*. High exposures are shown for the purpose of visualization of the high molecular weight cross-linked bands; under these conditions the intensities of the monomeric bands are underrepresented. Cross-linked products containing Mfn2 and Stoml2 are indicated with *red arrows*, Mfn1 monomers with a *black arrow*, Mfn2 monomers with a *green arrow*, intramolecularly cross-linked Mfn2 with a *purple arrow*, cross-linked product containing Stoml2, but not Mfn2, with an *orange arrow*, Stoml2 monomers with a *blue arrow*, and an unidentified band cross-reacting with anti-Mfn2 antibodies with an *asterisk*.

membrane. We concluded that Stoml2 is imported into mitochondria in a membrane potential-dependent manner and that the import is accompanied by proteolytic processing. The sublocalization and import experiments together suggest that Stoml2 is an intermembrane space protein.

Stoml2 Forms a High Molecular Weight Complex with Mfn2—To control for potential artifacts of FA cross-linking, co-immunoprecipitation of digitonin cell lysates with anti-Mfn2 antibodies followed by immunoblotting with anti-Stoml2 antibodies was performed (Fig. 6). Anti-Mfn2 antibodies, but not preimmune serum or anti-Mfn1 antibodies, co-precipitated Stoml2 protein, thus confirming the occurrence of a specific Mfn2-Stoml2 complex.

To address the question of whether Stoml2 is present in high molecular weight Mfn2-containing oligomers detected by FA

cross-linking, experiments similar to those described in Fig. 1 were carried out (Fig. 7). It should be noted first that anti-Stoml2 antibodies did not cross-react with Mfn2 and that anti-Mfn2 antibodies did not cross-react with Stoml2 (Fig. 7 and data not shown). In control cells (mock), immunoblotting with anti-Mfn2 antibodies or with anti-Stoml2 antibodies detected identical ladders of FA-cross-linked high molecular weight oligomers (Fig. 7*A*, *red arrows*). These immunoreactive ladders were not observed in the absence of the cross-linker. The anti-Mfn2 antibodies weakly cross-reacted with unidentified ~52- and 47-kDa proteins (Western blot data not shown), migrating as a single band in Fig. 7 (marked by an *asterisk*). These proteins, however, could not contribute to the high molecular weight cross-linked oligomers, because they were absent in mitochondrial fractions, which, upon FA treatment, gave a similar pattern of the high molecular weight oligomers (data not shown). Importantly, the pattern of high molecular weight cross-linked products was specifically detected with anti-Mfn2 or anti-Stoml2 antibodies but not with anti-actin, anti-α-tubulin, anti-Opa1, or anti-prohibitin antibodies (data not shown).

When cells were treated for 4 days with siRNA directed against STOML2 mRNA, which reduced the Stoml2 protein levels to ~20% compared with the mock-treated controls, the FA-cross-linked products detected with both anti-Mfn2 and anti-Stoml2 antibodies were dramatically decreased (Fig. 7*A*, *red arrows*). When HeLa cells were transfected with a Stoml2 expression plasmid, which led to an estimated 4–5-fold increase in Stoml2 protein levels, the FA-cross-linked products detected with both anti-Mfn2 and anti-Stoml2 antibodies were dramatically increased (Fig. 7*B*, *red arrows*). Under the conditions of Stoml2 down-regulation and also Stoml2 up-regulation, the endogenous levels of Mfn2 did not change (Fig. 7, *A* and *B*, and results not shown).

When cells were transfected with Mfn2 expression plasmid, which led to an estimated 3–4-fold increase in the Mfn2 protein, the FA-cross-linked products detected with both anti-Mfn2 and anti-Stoml2 antibodies were not significantly increased. Instead, a smeary Mfn2-immunoreactive pattern was observed under these conditions, an indication of nonspe-

A

B
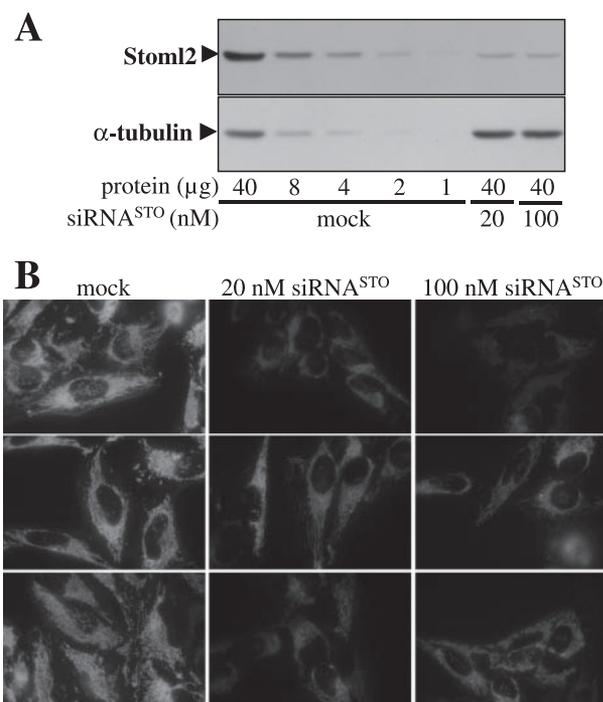
FIGURE 8. **Stoml2 knockdown experiments reveal reduced mitochondrial membrane potential in HeLa CCL2 cells.** *A,* indicated amounts of mock-treated cells or cells treated with 20 nM siRNA^STOML2^ or with 100 nM siRNA^STOML2^ for 6 days were analyzed by Western blotting with anti-Stoml2 or anti-α-tubulin antibodies. *B,* sets of three representative images of cells treated as described in *A* and stained with 25 nM TMRE.

TABLE 1

FACS analysis of HeLa cells

Mock-treated HeLa cells or cells treated with siRNA^STOML2^, as described under "Experimental Procedures," were stained with 10 nM TMRE to equilibrium and analyzed by FACS.

siRNA^STOML2^	Fluorescence
	geometric mean
Mock	540.7
20 nM	453.1
100 nM	438.4

ero-oligomers. The quantification of the discrete high molecular weight cross-linked species (8% in the four major discrete species) in Fig. 1*B* supports this idea.

Among the cross-linked products detected by Stoml2 antibodies, but not by Mfn2 antibodies, one had an apparent molecular mass of ~75 kDa and could represent a Stoml2 dimer (Fig. 7, *orange arrows*). A larger cross-linked product, with a molecular mass of ~139 kDa, was immunoreactive with both Mfn2 and Stoml2 antibodies and therefore could represent a heterotrimer composed of one molecule of Mfn2 and two molecules of Stoml2. The higher molecular weight hetero-oligomers are regularly spaced from each other at ~50–70 kDa intervals.

What Is the Function of Stoml2?—Mitochondrial morphology is determined by a dynamic equilibrium of two opposing processes, mitochondrial fusion and mitochondrial fission. Depletion of Mfn2 leads to the disruption of the fusion process, and the consequent prevailing mitochondrial fission leads to a fragmented type of mitochondrial morphology (39). To address the function of Stoml2 in mitochondrial fusion, HeLa cells were treated with siRNA^STOML2^, and the effect on mitochondria, stained with the mitochondria-specific dye MitoTracker Red, was determined. An improved protocol of siRNA^STOML2^ treatment led to a dramatic decrease in Stoml2 protein levels (Fig. 8*A*). When these were normalized to the levels of α-tubulin, the 20 or 100 nM siRNA^STOML2^-treated cells had only 2–5% residual levels of Stoml2 as compared with mock-treated cells. However, under these conditions, no dramatic change of mitochondrial morphology in cells treated with siRNA^STOML2^ and stained with 50 nM MitoTracker Red was observed when compared with mock-treated cells (data not shown).

Although a reduction of Stoml2 protein levels does not appear to change mitochondrial morphology, we observed a clear decrease of MitoTracker Red fluorescence under these conditions of siRNA^STOML2^ treatment (data not shown). To address the question of whether the mitochondrial membrane potential is compromised if Stoml2 is down-regulated, HeLa cells were treated with siRNA^STOML2^ as described above and stained with the potentiometric dye TMRE (Fig. 8*B*). When compared with mock-treated HeLa cells, the cells treated with siRNA^STOML2^ exhibited a clear reduction of the mitochondria-associated TMRE fluorescence intensity, an indication of decreased mitochondrial membrane potential. In contrast, treatment of HeLa cells with siRNA^MFN2^, that led to a decrease of the Mfn2 levels to ~5%, did not noticeably affect the TMRE fluorescence intensity (data not shown). To confirm the specific effect of Stoml2 reduction on the mitochondrial membrane potential, mock-treated cells and cells treated with 20 or 100 nM siRNA^STOML2^ were stained with TMRE and analyzed by

cific cross-linked products probably resulting from Mfn2 over-expression (Fig. 7*B*). When HeLa cells were treated for 4 days with siRNA directed against MFN2 mRNA, which reduced the Mfn2 protein levels to ~5% when compared with mock-treated controls, the FA-cross-linked products generated by a 1- or 5-min FA treatment and detected with both anti-Mfn2 and anti-Stoml2 antibodies were not significantly decreased (Fig. 7*C*, *red arrows*). Under these conditions of Mfn2 down-regulation, we observed a strong up-regulation of Mfn1 (~2.8-fold). To address the question of whether Mfn1 can substitute for Mfn2 in the complexes with Stoml2, the cells were treated with siRNA^MFN1^, alone or in combination with siRNA^MFN2^, and then were analyzed as described above. However, neither down-regulation of Mfn1 alone or in combination with down-regulation of Mfn2 led to a significant decrease in the FA-cross-linked products detected with both anti-Mfn2 and anti-Stoml2 antibodies (Fig. 7*C*, *red arrows*). Thus, the residual Mfn2 after siRNA^MFN2^ treatment appears to be in amounts sufficient for the formation of the Mfn2-Stoml2 complex. Possibly Mfn2 is stabilized by its association with Stoml2 and therefore persists even after siRNA^MFN2^ treatment. Interestingly, under the conditions of Mfn1 down-regulation, we observed a significant up-regulation of Mfn2. Under the conditions of down-regulation of both Mfn1 and Mfn2, there was still about ~10% residual Mfn2 protein when compared with the mock-treated cells. The down-regulation and up-regulation experiments described above clearly demonstrate that Stoml2 is a limiting component of the Stoml2-Mfn2 complex. The Mfn2 down-regulation and up-regulation experiments suggest that only a small portion of the cellular Mfn2 is involved in forming the Mfn2-Stoml2 het-

FACS. Consistent with the previous observations, cells treated with 20 and 100 nM siRNA[STOML2] exhibited, respectively, an ~16% and an ~19% decrease in the geometric mean of TMRE fluorescence when compared with mock-treated cells (Table 1). Importantly, the treatment of HeLa cells with 20 or 100 nM siRNA[STOML2] did not affect the rate of oxygen consumption in intact cells driven by the endogenous substrates, the maximal rate of oxygen consumption in dinitrophenol-uncoupled mitochondria, or the rate of oxygen consumption driven by ascorbate/N,N,N',N'-tetramethyl-1,4-phenylenediamine in the presence of antimycin A (data not shown).

DISCUSSION

In the present work, we have identified a complex between the mitochondrial fusion mediator Mfn2 and Stoml2. The initial evidence for the occurrence of this complex was obtained by the use of *in vivo* cross-linking of HeLa cells with formaldehyde and immunoprecipitation with anti-Mfn2 antibodies. An ~42-kDa protein, which was specifically immunoprecipitated with Mfn2 antibodies, was then identified as Stoml2. The presence of an endogenous Mfn2-Stoml2 complex was subsequently confirmed by a co-immunoprecipitation experiment using cells lysed with digitonin. In this experiment, Stoml2 was detected by immunoblotting in the immunoprecipitate obtained with anti-Mfn2 antibodies. This interaction with Stoml2 appeared to be specific for Mfn2, because an immunoprecipitation with Mfn1 antibodies did not yield Stoml2.

The human Stoml2 gene had been cloned and characterized previously (33, 40). Stoml2 is a member of the stomatin super-family because of its stomatin consensus signature sequence (33). Human Stoml2 and stomatin have, however, very low overall homology (20% similarity at the amino acid level), and Stoml2 lacks the typical N-terminal transmembrane domain present in other stomatins. Previous studies have suggested that Stoml2 is associated with the plasma membrane and other cellular membranes, on the basis of immunocytochemistry of human erythrocytes (33). In our work, however, Stoml2 was found to co-localize with mitochondrial markers both in immunocytochemical and cell fractionation experiments, and we did not detect any plasma membrane staining in our immunocytochemical experiments. A sequence analysis of the Stoml2 N terminus using the MitoProt II program revealed the high probability (98%) of its carrying a mitochondrial targeting sequence. Indeed, we found that Stoml2 is efficiently imported into isolated mitochondria. The import is accompanied by proteolytic processing and is dependent on an intact mitochondrial membrane potential. Our results are supported by several proteomic studies reporting the presence of Stoml2 in mitochondria (35–37). In addition, hemagglutinin-tagged Stoml2 was found to be localized in mitochondria in one of these studies (35). Thus, our work has confirmed that Stoml2 is indeed a mitochondrial protein. The above discussed findings suggesting that Stoml2 is associated with the plasma membrane may reflect situations in specialized cells, such as erythrocyte ghosts, which lack mitochondria or need to be revisited.

Subsequently, we found that Stoml2 is associated with mitochondrial membranes and exhibits an integral membrane protein-like behavior. As mentioned above, sequence analysis of Stoml2 did not predict the presence of a transmembrane domain, and thus the nature of the Stoml2 attachment to the mitochondrial membranes is presently unclear. In this regard, it was suggested that membrane attachment of stomatin is mediated not only by its transmembrane domain but also by the recently discovered palmitoylation of stomatin (41). A similar post-translational modification of Stoml2 could also mediate its membrane association. In the present work, further sublocalization studies showed that Stoml2 was not sensitive to externally added protease in intact mitochondria, but it was sensitive in mitochondria treated with a low concentration of digitonin. This treatment produced the protease sensitivity of an intermembrane space marker but not of a mitochondrial matrix marker. Thus, our experiments have indicated that Stoml2 is localized in the mitochondrial intermembrane space. The characteristics of the import of Stoml2 into the mitochondria, discussed above, are typical of mitochondrial proteins localized in the matrix or the inner membrane or intermembrane space but not for proteins of the outer mitochondrial membrane. These characteristics and our data on the submitochondrial localization of Stoml2 suggest that Stoml2 is associated with the inner mitochondrial membrane and projects toward the intermembrane space. However, we cannot formally exclude the possibility that Stoml2 is associated with the outer mitochondrial membrane.

As far as the Mfn2-Stoml2 complex is concerned, immunoblotting of cell lysates prepared from FA-cross-linked cells detected a ladder-like pattern of products containing both Mfn2 and Stoml2. These FA-cross-linked products were strongly decreased in amount when the cellular Stoml2 was reduced by the use of siRNA treatment, whereas they were increased when cellular Stoml2 was overexpressed. However, the FA-cross-linked products were not significantly changed in amount when the Mfn2 protein levels were manipulated. These results suggest that Stoml2 is a limiting component of the Mfn2-Stoml2 complex and that only a small fraction of the cellular Mfn2 is present in the complex. Using formaldehyde cross-linking, we were not able to detect cross-linked products containing both Mfn1 and Mfn2 by Western blotting. This could be because of an insolubility of cross-linked Mfn1-Mfn2 complex or because of low endogenous levels of the Mfn1-Mfn2 complex. Indeed, this complex was detected only in overexpression systems (5, 9). However, a large scale preparation of proteins cross-linked to Mfn2 revealed, after reversal of the cross-linking, that about equal amounts of Mfn1 and Mfn2 were co-purified (Fig. 2*B*), strongly suggesting the existence of Mfn1-Mfn2 hetero-oligomers.

The immunoblotting of formaldehyde cross-linked cells with anti-Stoml2 antibodies (but not with anti-Mfn2 antibodies) detected an ~75-kDa product, which could represent a Stoml2 dimer. Higher molecular weight species of ~140 kDa, reactive with both antibodies, might represent a trimer consisting of two molecules of Stoml2 and one molecule of Mfn2. Interestingly, at least seven additional bands with regular spacing of ~50–70 kDa were detected with both antibodies. However, we predict that not all of the detected cross-linked species are present *in vivo* but rather that they were the result of incomplete cross-linking of the complex. Our observation that longer times

of the cross-linking reaction lead to a shift of the cross-linked products toward higher molecular weight species is consistent with this prediction. It is also possible that other proteins were present in the detected Stoml2-Mfn2 complex. Following this model, Stoml2 might form an important scaffold in the mitochondrial inner membrane with which the outer membrane fusion machinery interacts. Interestingly, in this regard, we observed a small but reproducible reduction of mitochondrial membrane potential in cells treated with siRNA[STOML2]. It is thus possible that a disturbed organization of the Stoml2 scaffold leads to a decrease in mitochondrial membrane potential.

With regard to the Stoml2 function in mitochondrial fusion, no obvious changes in mitochondrial morphology were detected when HeLa cells were treated with siRNA[STOML2], although these conditions reduced the Stoml2 protein levels to ~5% as compared with mock-treated cells. In a similar experiment, siRNA reduction of Mfn2 to ~5% also had no dramatic effect on mitochondrial morphology (data not shown). Thus, the negative results obtained with siRNA[STOML2] could result from a limitation of the siRNA technology, which does not always produce a complete depletion of the desired protein in the cell. Treatment of cells with siRNAs directed against both Mfn1 and Mfn2, however, caused a change in mitochondrial morphology characterized by punctate mitochondria (data not shown). In this light, the results of the siRNA[MFN2] experiment could be explained by overlapping functions of Mfn2 and Mfn1 in fusion as reported previously (5). Furthermore, it is also possible that a protein homologous to Stoml2 exists and substitutes for a possible Stoml2 function in mitochondrial fusion. Interestingly, a prohibitin homology domain can be detected within the Stoml2 amino acid sequence. Thus, perhaps, a prohibitin can substitute for Stoml2. Yeast prohibitins are mitochondrial proteins anchored in the inner mitochondrial membrane and projecting into the intermembrane space (42). They form large oligomeric complexes, which have been suggested to function as regulators of protein degradation by m-AAA protease or as molecular chaperones for mtDNA-encoded proteins (43–45). In addition, the formation of prohibitin ring structures with outer dimension of ~270 × 200 Å has recently been reported, and their scaffolding function in the mitochondrial inner membrane has been suggested (42). The ring structures of prohibitins may also be important for the maintenance of mitochondrial morphology. Previous reports demonstrated that, in the absence of prohibitins, fragmented mitochondria occur in yeast cells lacking mtDNA or in the *Caenorhabditis elegans* body wall muscle cells (46–48). Further work is needed to clarify the functional relevance, if any, of Stoml2 in the formation of an inner membrane scaffold and in the maintenance of mitochondrial morphology.

The coordination of the outer membrane and the inner membrane fusion events is not understood at present. The importance of the intermembrane space loop of the yeast mitofusin homolog Fzo1 for its interaction with the mitochondrial inner membrane and for normal mitochondrial morphology has been demonstrated previously (49). It was also suggested that this interaction is mediated via an unknown protein of the inner mitochondrial membrane. On the basis of the intermembrane space/inner membrane localization of Stoml2, we spec-

ulate that Stoml2 may interact with the intermembrane space loop of Mfn2. This loop contains a tryptophan residue (Trp-631 of Mfn2), highly conserved among mitofusin/Fzo proteins. Interestingly, it has been reported that COS7 cells expressing the Mfn2[W631P] mutant protein exhibit fragmented mitochondria, even though the localization of the mutant Mfn2 is unaffected (50).

Note Added in Proof—A new protein involved in mitochondrial fusion in mammalian cells has recently been identified (Choi, S. Y., Huang, P., Jenkins, G. M., Chn, D. C., Schiller, J., and Frohman, M. A. (2006) *Nat. Cell Biol.* **8,** 1255–1262).

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