ABSTRACT  Mitochondrial fission and proteins vital to this process play essential roles in apoptosis. Several mitochondrial outer membrane proteins, including mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff) and mitochondrial dynamics of 51 kDa protein (MiD51), also known as mitochondrial elongation factor 1 (MIEF1), have been reported to promote mitochondrial fission by recruiting the GTPase dynamin-related protein 1 (Drp1). However, it remains unclear how these fission factors coordinate to control apoptotic mitochondrial fission. Molecular studies have suggested the existence of interaction between Mff and Drp1, but fundamental questions remain concerning their function. In the present study, we reported that the phosphorylation status of Drp1-Ser637 was essential for its interaction with Mff. UV stimulation induced a decrease in cytoplasmic and mitochondrial Drp1 phosphorylation on Ser637 and enhanced the interaction between Drp1 and Mff, resulting in mitochondrial fragmentation. Simultaneously, the interaction increased markedly between Fis1 and MiD51/MIEF1, whereas the interaction between Drp1 and MiD51/MIEF1 decreased significantly after UV irradiation, which suggests that Fis1 competitively binds to MiD51/MIEF1 to activate Drp1 indirectly. Moreover, Mff-Drp1 binding and Mff-mediated recruitment of Drp1 to mitochondria did not require Bax during UV stimulation. Our study revealed a novel role of Mff in regulation of mitochondrial fission and showed how the fission proteins are orchestrated to mediate the fission process during apoptosis.—Zhang, Z., Liu, L., Wu, S., Xing, D. Drp1, Mff, Fis1, and MiD51 are coordinated to mediate mitochondrial fission during UV irradiation–induced apoptosis. FASEBJ. 30, 466–476 (2016). www.fasebj.org

Key Words: MIEF1 · mitochondrial fragmentation · GTP-binding · oligomerization · Bax

Mitochondria are the primary energy-generating system of vital importance for cell survival (1). Changes in mitochondrial morphology are tightly regulated by the balanced fusion and fission processes. Fusion helps mitochondria to compensate for one another’s defects in the face of cellular stress (2). Fission not only creates new mitochondria, but also eliminates the damaged mitochondria to preserve the health of the mitochondrial network and ensure mitochondrial quality control (3). Alterations in mitochondrial dynamics underlie various human diseases, including cancer and neurologic and cardiovascular diseases (4, 5). Therefore, disclosing mitochondrial dynamics–related signaling pathways help us to better understand roles of mitochondria involved in related disease and to discover strategies for preventing the related diseases. Several mitochondrial outer membrane–anchored proteins, including mitochondrial fission protein 1 (Fis1) (6–8), mitochondrial fission factor (Mff) (9, 10), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51, respectively) (11, 12), have been proposed to promote mitochondrial fission by recruiting the GTPase dynamin-related protein 1 (Drp1) in mammals. However, fundamental questions remain concerning their function.

Mitochondrial fission is a complex process initiated by the recruitment of Drp1 to the mitochondrial surface. Then, Drp1 assembles into constrictive ringlike multimers around the organelles and finally drives the fission process through a GTP hydrolysis–dependent mechanism (13). It has been proposed that Drp1 translocates to prospective fission sites of mitochondria through Fis1 and Mff (8, 10, 14). However, there is still a debate over the proposed function of Fis1 in Drp1 recruitment. Unlike Mff, as has been observed in previous studies, deletion of Fis1 does not affect Drp1 distribution (10, 15). On the contrary, a recent report found that Fls1-null mouse embryonic fibroblasts (MEFs) have reduced Drp1 puncta at the mitochondria (16). Similar to Mff, MiD51 [also termed mitochondrial elongation factor 1 (MIEF1)], and the variant MiD49 (also called MIEF2) recruits Drp1 to the mitochondrial outer membrane (MOM) (12), but there is conflicting evidence concerning their mechanism of action. As Drp1 receptors, knockdown of both MiD49/MIEF2

Abbreviations: BP, band-pass; BSA, bovine serum albumin; co-IP, coimmunoprecipitation; Drp1, dynamin-related protein 1; Em., emission; Ex., excitation; FA, formaldehyde; Fis1, mitochondrial fission protein 1; LP, long-pass; MEF, mouse embryonic fibroblast; Mff, mitochondrial fission factor; MiD49, mitochondrial dynamics protein; MIEF1, mitochondrial elongation factor 1; MOM, mitochondrial outer membrane; RNAi, interference RNA

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and MiD51/MIEF1 resulted in an irregular distribution of the network and fused mitochondria (12). More recently, MiD49/MIEF2 and MiD51/MIEF1 were shown to mediate Drp1-dependent mitochondrial fission in Fis1/Mff double-KO MEFs (16). In addition, ADP-bound MiD51/MIEF1 assembles Drp1 into spirals and promotes Drp1 GTP hydrolysis, supporting a role in mitochondrial fission (17). Paradoxically, overexpression of either MiD49/MIEF2 or MiD51/MIEF1 causes increased recruitment of Drp1 to the mitochondrial surface, but inhibits Drp1 activity, thus executing a negative effect on mitochondrial fission (11, 12, 16, 18). Based on these studies, it appears that MiD51/MIEF1 or MiD49/MIEF2 alone acts as a suppressor to sequester Drp1 and inhibits Drp1-mediated fission. Furthermore, the functional properties of Drp1 can be regulated in part by posttranslational modifications, including phosphorylation (19–21), sumoylation (22), and ubiquitination (23, 24). Drp1 phosphorylation at Ser616 by the CDK1/cyclin B complex leads to mitochondrial fission during mitosis (20). In contrast, PKA-mediated phosphorylation at Ser637 inactivates Drp1 fission, but this effect can be counteracted by calcineurin (19, 21). So far, the impact of Ser637 phosphorylation has gained prominence in mitochondrial function. Despite much effort, understanding of the precise mechanisms that coordinate the complex process of mitochondrial fission remains limited. Further understanding of the machinery involved in mitochondrial dynamics, including the regulation of Drp1 function, is therefore needed.

Mitochondrial fission is considered to be universally associated with the initiation of apoptosis (15, 25). Under physiologic conditions, mitochondria are tubular in shape, but they undergo extensive fragmentation upon apoptotic stimulation (25). Studies have suggested that the Bcl-2 family member Bax may regulate apoptosis through morphogenesis machinery (26, 27). During apoptosis, Drp1 recruitment is enhanced, resulting in mitochondrial fragmentation (28). Simultaneous with mitochondrial fragmentation, activated Bax clusters coalesce at Drp1-containing fission sites, implying that they may intersect in membrane remodeling (22, 29). Besides, activated Bax is essential for MOM permeabilization, which is accompanied by Drp1-mediated mitochondrial fission (30). Bax permeabilizes the MOM only when it is oligomerized, and recent data demonstrate that Drp1 has been involved in tBid-induced Bax oligomerization with membrane remodeling (31, 32), suggesting a mechanistic link between Drp1-dependent mitochondrial fission and Bax-dependent apoptosis. However, how the fission proteins and apoptosis-related proteins are orchestrated to mediate the fission process during apoptosis warrant further investigation.

UV irradiation is a potent carcinogen that can impair cellular functions by directly damaging DNA to induce apoptosis. Increasing evidence suggests that a mitochondrion-dependent pathway plays an important role in UV-induced apoptosis (33, 34). Given this, it is perhaps not surprising that abnormal mitochondrial dynamics have been implicated in UV-induced apoptosis, but the underlying mechanisms remain unclear. In this study, we uncover the machinery involved in mitochondrial dynamics, elucidating the role of Mff in regulating Drp1 function and mitochondrial fission. Our study provides new mechanistic insight on how Mff functions in mitochondrial fission and how the fission proteins are orchestrated to mediate fission process during UV-induced apoptosis.

MATERIALS AND METHODS

Cell culture and transfections

The human lung adenocarcinoma cell line ASTC-a-1 was obtained from the Department of Medicine, Jinan University (Guangzhou, China). ASTC-a-1 and COS-7 cells were cultured in DMEM (Thermo Fisher–Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) in 5% CO2 at 37°C. Transfections were performed with Lipofectamine 2000 reagent (Thermo Fisher–Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Cells were examined 36–48 h after transfection.

Plasmids and RNA interference

The cDNA of Mff was amplified by PCR from ASTCa-1 cell total RNA by RT-PCR, with the primers 5’-CCCAAGCTTCCATTGCGAACAAATTAGTGCAGAGGATGAdTdT-3’ and 5’-GGCG GATCCAGTCGACCCCATGGTCATGTATTCAA-3’. The PCR product was digested with EcoRI and cloned into pEGFP-C1 vector to generate GFP-Mff and GFP-Mff. For creation of N-terminal truncated Mff constructs, the Mff ORF was amplified with the common reverse primer 5’-CCCAAGCTTCCAGGGCGCAAAACCCAGCATTGACCT-3’ for GFP-Mff [32–219]; 5’-CCCAAGCTTCCATTGCAAGTCGGAGGACCGAAGA-3’ for GFP-Mff [51–219]; 5’-CCCAAGCTTCCATTGCAAGTCGGAGGACCGAAGA-3’ for GFP-Mff [130–219]; and 5’-CCCAAGCTTCCATTGCAAGTCGGAGGACCGAAGA-3’ for GFP-Mff [199–219]. The DNA products were digested with HindIII and BamHI and cloned into the pEFGP-C1 vector to produce fusion to GFP. For GFP-Mff [199–219], the DNA products were cloned into the EcoRI and BamHI site of pEFGP-C1. All constructs were verified by sequencing.

Mff, Drp1, Bax, and MiD51/MIEF1 suppression were accomplished using Mff shRNA, Drp1 shRNA, and Bax shRNA constructs and siMiD51/MIEF1, respectively. The target sequences for the Mff, Drp1, and Bax were synthesized as follows: shMff: 5’-CGCTGACCTGAAAGAGGAdTdT T-3’; shDrp1: 5’-CTCAGGGTATCCATTGACCTGAGAATGATTCCAAATGTCCTAAATCACAG-3’; shBax: 5’-AACATGAGGTCTGGAGAGATGAdTdT T-3’; and siMiD51/MIEF1: 5’-AGATTCGACCTGACCTGAAAGAGGAdTdT T-3’.

Antibodies and other reagents

The antibodies used in this study included rabbit polyclonal anti-Drp1 (H-300), anti-β-actin, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA); purified mouse anti-DLP1/Drp1 (BD Transduction Laboratories, Lexington, KY, USA); anti-COXIV, rabbit polyclonal anti-Drp1 (Ser637), and rabbit monoclonal anti-green fluorescent protein (GFP) (Cell Signaling Technology, Danvers, MA, USA); Mff, MiD51/MIEF1, and mouse monoclonal anti-His (Proteintech Group, Chicago, IL, USA); and Fis1 (GeneTex, Irvine, CA, USA). The fluorophore probe used to label mitochondria was MitoTracker Deep Red 633 (100 nM; Thermo Fisher–Invitrogen). UV treatment was performed as we reported previously (33, 34).
GTP-binding assays were performed as described, with slight modifications (11). In brief, 48 h after transfection, ASTC-a-1 cells were rinsed in ice-cold PBS and collected in 300 μl GTP-binding buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton-X 100, 5 mM MgCl2, 2 mM PMSF, protease inhibitor cocktail, and 1 mM DTT). Samples were sonicated and centrifuged, and the supernatant was collected. The cleared lysates were incubated with 30 μl of GTP-agarose beads (Sigma-Aldrich, St. Louis, MO, USA) at 30°C for 1 h. The beads were washed 3 times with GTP-binding buffer. After the final wash, bound protein was eluted from the beads by boiling them in sample buffer. The proteins were resolved by SDS-PAGE and analyzed by Western blot analysis with anti-Drp1 antibody.

Immunofluorescence

Cells on 22-mm culture glasses were transfected with DsRed-Mit to label mitochondria. The transfected cells were fixed with 3.7% paraformaldehyde for 10 min at room temperature, permeabilization with 0.2% Triton X-100 in PBS for 15 min, and blocked with 1% bovine serum albumin (BSA) for 45 min, followed by overnight incubation with anti-Drp1 (1:200; Santa Cruz Biotechnology) antibody at 4°C, 3 washes with PBS, and 2 h incubation at room temperature with FITC-conjugated secondary antibodies (1:50; Proteintech Group, Chicago, IL, USA). Images were acquired with a laser scanning confocal microscope (LSM 510 Metz; Zeiss).

Real-time quantitative PCR analysis

Total RNA was extracted from cells using the RNAiso Plus (Takara, Shiga, Japan). ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) was used to synthesize cDNA. The following primers were used: Drp1: 5'-GATGCCATAGTGGAGTGCTGGAC-3' and 5'-TTTTGTGTGTCATAACACCTCTCC-3'; Mff: 5'-CCAAACCGTGACCTGGAAAC-3' and 5'-TTTTGTGTGTCATAACACCTCTCC-3'; Mff: 5'-AGGATGCAATGGCATGCCGAC-3' and 5'-CCGATCTGACCTGGAAAC-3'; Mff: 5'-CAAGGAACCTGGAGGCCTTAC-3' and 5'-GGAGAGAATGCTGGACATGCTAG-3'; and β-actin: 5'-CCAGAGGAGAATGCTGGACATGCTAG-3'. The cDNA was loaded into capillary tubes with SybrGreen qPCR Master Mix and then incubated in the LightCycler for an initial denaturation at 95°C for 10 s, followed by 40 cycles, each cycle consisting of 95°C for 5 s, 60°C for 10 s, and 72°C for 10 s. β-Actin was detected to normalize gene expression.

Subcellular fractionation

Subcellular fractions were prepared from the cells according to a published method (39).

Western blot assays

Western blot analysis was performed with some modifications (40). At the indicated time after different treatments, cells were washed with ice-cold PBS and prepared in lysis buffer for 30–60 min on ice. Equivalent proteins were loaded on SDS-PAGE, transferred to the PVDF membrane (EMD Millipore, Billerica, MA, USA), and blotted with the indicated primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies. Detection was performed using the Odyssey infrared imaging system (Li-Cor, Lincoln, NE, USA).

Statistical analysis

All assays were repeated independently a minimum of 3 times. Data represent the means ± SD. Statistical analysis was performed with paired Student’s t test. Differences were considered statistically significant at P < 0.05.
RESULTS

Mff regulates mitochondrial fission through Drp1

To define the Mff region responsible for mitochondrial dynamics, full-length and N-terminally truncated Mff constructs (Fig. 1A) were expressed in ASTC-a-1 or COS-7 cells. When expressed in ASTC-a-1 and COS-7 cells, GFP-Mff localized to mitochondria (Fig. 1B), which was consistent with recent studies showing the mitochondrial localization of endogenous Mff (9, 10), confirming that the fused Mff constructs did not affect its subcellular distribution. Four GFP-fused, truncated Mff constructs (GFP-Mff[32–219], GFP-Mff[51–219], GFP-Mff[130–219], and GFP-Mff[199–219]) were also localized to mitochondria (Fig. 1C), confirming that the intact C-terminal structure of

![Figure 1](image-url)

**Figure 1.** Mff regulates mitochondrial fission in a Drp1-dependent manner. A) GFP-tagged Mff fusion constructs. B) Colocalization of Mff with mitochondria. ASTC-a-1 and COS-7 cells expressing GFP-Mff (green) were stained with MitoTracker (red) to visualize the mitochondria. C) Distribution of N-terminal truncated, GFP-tagged Mff constructs. ASTC-a-1 cells were transiently transfected with the constructs. After incubation for 36 h, the cells were stained with MitoTracker and analyzed by confocal microscopy. D) Percentages of ASTC-a-1 cells with the indicated mitochondrial morphologies in nontransfected and GFP-, GFP-tagged full-length–, or N-terminal–truncated–transfected cells at 36 h after transfection. The 3 panels represent the 3 mitochondrial morphologies used for scoring the different mitochondrial phenotypes. *P < 0.05 vs. GFP-Mff transfected cells. E) Knockdown of Mff induces mitochondria elongation. ASTC-a-1 cells transfected with control RNAi or Mff RNAi and stained with MitoTracker. Western blot analysis shows protein levels in RNAi-transfected cells. *P < 0.05 vs. control. F) Mff-induced mitochondrial fission requires intact Drp1 function. COS-7 cells were transiently cotransfected with GFP-Mff and shDrp1 and analyzed by confocal microscopy. The data in (D–F) are means ± SD of 3 independent experiments and were collected from 150–200 cells per treatment. Magnified areas provide more detailed information. Scale bars, 10 μm.
Mff, but not the N-terminal region, was sufficient to target mitochondria.

The basic mitochondrial morphology in nontransfected and vector-transfected ASTCα-1 cells was a tubular network (Fig. 1D). Mff overexpression induced mitochondrial fission (Fig. 1D). Conversely, deletion of the first amino-terminal repeat of Mff (GFP-Mff32–219 and GFP-Mff51–219) markedly reduced the number of cells with short, fragmented mitochondria (Fig. 1D), and greater reductions of the fragmented phenotype was observed upon further deletions of 2 short amino-terminal repeats of Mff (GFP-Mff130–219). Minor mitochondrial fragmentation was detected in cells expressing GFP-Mff199–219 that lacked the entirely N-terminal region (Fig. 1D). Together, these findings indicate that the N-terminal region of Mff is essential for mitochondrial fission.

To further detect the role of Mff in Drp1-mediated mitochondrial fission, we used interference (RNAi) to inhibit Mff expression in ASTCα-1 cells. Knockdown of Mff frequently exhibited elongated mitochondrial network (Fig. 1E) (9, 10). GFP-Mff overexpression in control RNAi-transfected COS-7 cells resulted in mitochondrial fragmentation (Fig. 1A). However, the tubular mitochondrial structures were not affected by the expression of GFP-Mff in Drp1-RNAi–transfected COS-7 cells (Fig. 1F). Taken together, in agreement with the previous study (10), our data indicate that Mff regulates mitochondrial fission in a Drp1-dependent manner.

### Mff increases Drp1’s GTP-binding activity

Co-IP with full-length and deletion of Mff mutants expressed in ASTCα-1 cells showed that the 2 short amino-terminal repeats were essential for Mff’s interaction with Drp1 (Fig. 2A), further confirming the recent findings that Mff binds via its N-terminus directly to Drp1 (10). Although Mff and Drp1 interact with each other, it is unclear whether this interaction could alter Drp1’s GTP-binding ability and oligomerization. Mff overexpression increased the GTP binding of both endogenous Drp1 and exogenously expressed HA-Drp1, in an assay that measures GTP-binding activity in intact cells, as shown by pull-down experiments using GTP-conjugated beads, suggesting that Mff affects the GTPase activity of Drp1 via an increase of its GTP-binding ability (Fig. 2B, C). Although overexpression of human Fis1 did not affect the GTP binding of Drp1, indicating that Mff and human Fis1 activate the fission reaction in different ways. However, we did not find significant influence on the extent of oligomerization of endogenous Drp1 in GFP-Mff–transfected cells compared with that in GFP-transfected cells (Fig. 2D, E).

**Figure 2.** Mff increases Drp1’s GTP-binding activity without affecting the oligomerization of Drp1. A) Mff interacts with Drp1 via the N-terminal region. ASTCα-1 cells were transfected with the indicated plasmids for 36 h. After a brief *in vivo* cross-linking, cells were homogenized and whole-cell extract (WCE) was subjected to IP by anti-Drp1 antibody. Western blot analysis was performed to detect Drp1 or GFP in the IP complexes and WCE. B) Drp1 GTP-binding activity was measured by pull-down experiments using GTP-conjugated beads in ASTCα-1 cells transfected with the indicated plasmids. C) Quantitative data are provided in the histogram. *P < 0.05 vs. indicated group. D) Mff overexpression does not affect the oligomerization of endogenous Drp1. Cytosolic extracts from cells overexpressing GFP or GFP-Mff were cross-linked with BS3 at the indicated concentrations, resolved by SDS-PAGE, and immunoblotted with Drp1 antibodies. Chemical cross-linking with the BS3 showed a prominent cross-linked product at a molecular mass of ~280 kDa. E) Quantitative data are provided in the histogram.
conclusion, these results suggest that Mff increases Drp1’s GTP-binding affinity without affecting the oligomerization of Drp1.

**Dephosphorylation of Drp1-Ser<sup>637</sup> is essential for its interaction with Mff**

It has been reported that Ser<sup>637</sup> phosphorylation of Drp1 inhibits its translocation to mitochondria and thus prohibits the mitochondria from dividing. To test whether the Mff-induced relocation of Drp1 to mitochondria and mitochondrial fission depend on the phosphorylation status of Drp1, we expressed wild-type Drp1-YFP, the phosphorylation-deficient Drp1 Ser<sup>637</sup>A-YFP mutant, and the phosphomimetic Drp1 Ser<sup>637</sup>D-YFP mutant in the presence of CFP-Mff or not, and found that mutagenesis of Ser<sup>637</sup> had a major impact on subcellular localization of Drp1 and mitochondrial shape (Fig. 3A). Expression of wild-type Drp1 and Drp1 Ser<sup>637</sup>D-YFP revealed a predominantly cytoplasmic distribution, whereas Drp1 Ser<sup>637</sup>A-YFP was in part localized on mitochondria (Fig. 3A and Supplemental Fig. S1). Coexpression with CFP-Mff led to the recruitment of wild-type Drp1 and Drp1 Ser<sup>637</sup>A-YFP to the mitochondria, where they colocalized with CFP-Mff (Fig. 3B). However, Drp1 Ser<sup>637</sup>D-YFP did not translocate to the mitochondria, even in the presence of CFP-Mff (Fig. 3B and Supplemental Fig. S2). Statistical analysis data demonstrated that CFP-Mff overexpression promoted Drp1 mitochondrial translocation by the increase in R from 0.05 to 0.67 in Drp1-YFP-transfected cells and from 0.35 to 0.83 in Drp1-Ser<sup>637</sup>A-YFP-transfected cells, indicating the colocalization between Drp1 and Mff (Supplemental Figs. S1 and S2). However, the R did not increase in the Drp1-Ser<sup>637</sup>D-YFP–transfected cells when cotransfected with CFP-Mff. Furthermore, co-IP showed

![Figure 3. Dephosphorylation of Drp1-Ser<sup>637</sup> is necessary for its interaction with Mff. A) Confocal images of ASTC-a-1 cells transfected with Drp1-YFP, dephosphomimetic Drp1 Ser<sup>637</sup>A-YFP mutant, or phosphomimetic Drp1 Ser<sup>637</sup>D-YFP mutant alone. B) ASTC-a-1 cells cotransfected with CFP-Mff and Drp1-YFP, Drp1-Ser<sup>637</sup>A-YFP, or Drp1 Ser<sup>637</sup>D-YFP. Cells were stained with Mito-Tracker Red to visualize mitochondria. Scale bars, 10 μm. C) Representative Western blot and quantification analyses (D) of the binding of Myc-Mff to Drp1-YFP, Drp1-Ser<sup>637</sup>A-YFP, or Drp1-Ser<sup>637</sup>D-YFP was analyzed by Co-IP with anti-Mff agarose, followed by Western blot analysis with the indicated antibodies. *P < 0.05.
that Mff bound to wild-type Drp1-YFP and Drp1-Ser637A-YFP, and this binding was significantly decreased between Drp1-Ser637D-YFP and Mff (Fig. 3C). In sum, these data show that the phosphorylation status of Drp1-Ser637 is essential for its interaction with Mff.

Mff, Fis1, MiD51/MIEF1, and Drp1 are orchestrated to mediate fission process during UV-induced apoptosis

Mitochondrial fission machinery actively participates in the process of apoptosis. However, it remains unclear how the fission proteins are orchestrated to mediate the fission process during apoptosis. Using UV irradiation as the apoptotic stimulus, we first measured the expression pattern of mitochondrial fission proteins treated with 120 μJ/cm² UV irradiation. Western blot analysis revealed significant changes in the levels of fission proteins: Mff and MiD51/MIEF1 levels were significantly reduced after UV treatment. Unlike Mff and MiD51/MIEF1, Fis1 levels were slightly but significantly elevated after UV treatment, and Drp1 levels were significantly increased after UV irradiation compared to controls (Fig. 4A, B). Real-time qPCR studies confirmed a significant increase in Fis1 and Drp1 mRNA levels by UV irradiation, but no obvious changes in mRNA levels of MiD51/MIEF1 and Mff (Fig. 4C). Drp1 in mammalian cells is a major cytosolic enzyme and it is mitochondrial Drp1 that participates in mitochondrial fission. Our results showed that Drp1 was recruited to mitochondria after UV irradiation (Fig. 4D, E). We then examined the effect of UV on phosphorylation of Drp1 at Ser637 and found that exposure of cells to UV stimulus resulted in a significant decrease in the levels of cytoplasmic and mitochondrial phospho-Drp1 at Ser637 (Fig. 4F).

To further examine the role of Mff in UV-induced mitochondrial fragmentation, Co-IP was performed to detect the interaction between Mff and Drp1 during UV stimulation. We found that the Mff–Drp1 complex increased markedly after UV irradiation (Fig. 4F, G). In addition, we did not detect a significant interaction between Fis1 and Drp1 (Fig. 4F). These results suggest that Mff promotes mitochondrial fission by directly interacting with Drp1 during UV stimulation. MiD51/MIEF1 has been proposed as the receptor for Drp1-dependent fission but it inhibits Drp1’s activity. Thus, MiD51/MIEF1 exerts a negative effect on mitochondrial fission. Studies have also suggested an interaction between MiD51/MIEF1 and Fis1 (11). We therefore speculated that Fis1 promotes Drp1-mediated mitochondrial fission indirectly by competitive binding to MiD51/MIEF1. To test this possibility, co-IP was performed to detect the interactions of MiD51/MIEF1, Mff, Fis1, and Drp1 in response to UV irradiation. We found that Fis1–MiD51/MIEF1 interaction increased markedly, whereas the amount of MiD51/MIEF1 binding to Drp1 declined markedly after UV irradiation (Fig. 4H, I). In addition, we did not detect the interaction between MiD51/MIEF1 and Mff (Fig. 4H). Furthermore, to test whether the Fis1–MiD51/MIEF1 interaction was the nonspecific recovery in IP, we used RNAi of MiD51/MIEF1 and IP using Fis1 antibody and found that the interaction was not via non-specific recovery in IP (Fig. 4I). Together, these results demonstrate that Fis1, but not Mff, promotes Drp1-dependent mitochondrial fission indirectly by competitive binding to MiD51/MIEF1 after UV stimulation.

Bax is dispensable for Mff-Drp1 interaction in response to UV stimulation

Studies have demonstrated that overexpression of Bax promotes mitochondrial fragmentation upon apoptotic stimulation (28, 41, 42). Another study, however, reported that Bax deficiency does not prevent mitochondrial fragmentation during apoptosis (26). In the present study, we found that overexpression of Bax increased Drp1 accumulation in mitochondria in response to UV stimulation (Fig. 5A). Based on the former data, we wondered whether Bax-induced Drp1 recruitment would correlate with an increased Mff–Drp1 interaction during UV stimulation. As shown in, Knockdown of Bax did not cause alterations in the Mff–Drp1 interaction (Fig. 5B), suggesting that Bax-induced Drp1 recruitment and Mff-dependent Drp1 recruitment are separable events during UV stimulation. Subsequently, we examined the role of Bax in Mff-mediated recruitment of Drp1 during UV stimulation. We conducted subcellular fractionation experiments from GFP-Bax–expressing ASTC-a1 cells. The results showed that the levels of mitochondrial Drp1 significantly decreased in Mff-knockdown cells after UV irradiation (Fig. 5C). In summary, our results show that Mff–Drp1 binding and Mff-mediated recruitment of Drp1 to mitochondria do not require Bax to respond to UV stimulation.

DISCUSSION

Because mitochondrial fission machinery actively participates in the process of apoptosis, a considerable amount of research has been concentrated on the basic molecular mechanisms underlying mitochondrial fission (1, 43, 44). Growing evidence has reported mechanisms that regulate the membrane constriction and fission activity of Drp1. Key mechanistic questions are how Drp1 is recruited to the prospective fission sites of mitochondria. In mammals, the recruitment is achieved by several membrane receptor proteins, including Fis1 and Mff. However, the general role of Fis1 in mitochondrial fission has been questioned recently; for example, conditionally knocked out Fis1 in human HCT116 cells does not contribute to defective mitochondrial fission (10). Conversely, analysis of Fis1-null MEFs indicated a clear but minor role of Fis1 in Drp1 recruitment and mitochondrial fission (16). It appears that the relative importance of Fis1 vs. Mff may depend on the particular cell type. Given the powerful effects of Mff in Drp1 recruitment, our work has confirmed prior results (10) and extended it by demonstrating that Mff regulates mitochondrial fission via increasing Drp1’s GTP-binding activity without affecting the oligomerization of Drp1. Although previous reviews by Mihara’s group (45) have suggested that Mff facilitates membrane scission by promoting the self-assembly of Drp1. In this regard, our finding seems to be a bit contradictory with the previous review by Mihara’s group. We speculated that as-yet unknown Mff-interacting proteins may affect the assembly of the fission
Figure 4. Mff, Drp1, Fis1, and MiD51/MIEF1 are orchestrated to mediate fission process after UV irradiation. A) Representative Western blot of the expression levels of Mff, Drp1, Fis1, and MiD51/MIEF1 in ASTC-a-1 cells at 0, 3, and 6 h after 120 mJ/cm² UV irradiation. B) Quantitative data are provided in the histogram. **P < 0.01 and *P < 0.05 vs. indicated group. C) Real-time PCR analysis of mitochondrial fission factors by UV irradiation. mRNA levels were determined relative to β-actin. Data were from 3 independent experiments. *P < 0.05 vs. indicated group. D) Immunofluorescence analysis for the distribution of endogenous Drp1 after UV irradiation. ASTC-a-1 cells were transfected with DsRed-Mit (red) to visualize mitochondria before fixation and stained with anti-Drp1 followed by FITC-conjugated secondary antibodies (green) before (upper) at 6 h after UV irradiation. Magnified areas provide more detailed information. Scale bars, 10 µm. E) Changes in subcellular localization and modification of Drp1 during UV stimulation. Representative Western blots of Drp1 and phospho-Drp1 (Ser637) in the mitochondrial (Mito) and cytosolic (Cyto) fraction of UV-treated ASTC-a-1 cells. β-Actin and Cox IV were used as control markers for Cyto and Mito, respectively. F) After a brief in vivo cross-linking, the ASTC-a-1 cells were homogenized. Total cell lysates were then subjected to IP with the anti-Drp1 antibody, and the immunoprecipitates were analyzed by Western blot with anti-Mff and anti-Fis1 antibodies. G) Quantitative data are provided in the histogram. *P < 0.05 and **P < 0.01 vs. indicated group. H) After a brief in vivo cross-linking, Co-IP with an anti-MIEF1 antibody was used to pull down MIEF1, and Western blot analysis was performed to detect Drp1, Fis1, Mff, and MIEF1 in the IP complexes and WCE. J) Quantitative data are provided in the histogram. *P < 0.05 and **P < 0.01 vs. indicated group. J) ASTC-a-1 cells were transfected with indicated plasmids for 48 h and then treated with UV irradiation or not. After a brief in vivo cross-linking, cells were homogenized and WCE was subjected to IP by anti-Fis1 antibody. Western blot analysis was performed to detect indicated proteins the IP complexes and WCE. *P < 0.05.
machinery, leading to Drp1-dependent membrane constrictions and eventually to membrane scission. More important, we for the first time reveal that the phosphorylation status of Drp1-Ser637 is critical for its interaction with Mff.

Unlike Mff and Fis1, MiD51/MIEF1 was thought to act as a Drp1 suppressor protein that also recruits Drp1 to the mitochondrial surface (11). Whereas each of them has its distinct functions, they are all connected to mitochondrial fission, sometimes in a tissue-specific manner (10). Although the fission proteins function at steady state, their properties are likely to be altered to accommodate the apoptotic fission pathway. However, little is known regarding how these different fission proteins communicate with each other and coordinate mitochondrial fission during apoptosis. It has been reported that UV stimulation can impair cellular functions by directly damaging DNA to induce apoptosis. Our previous studies showed that a mitochondrion-dependent pathway plays an important role in UV-induced apoptosis (33, 34). Using UV irradiation as the apoptotic stimulus, we observed that UV stimulation significantly affected the protein level of mitochondrial fission proteins, implicating the role of mitochondrial fission in UV-induced apoptosis. Aside from the increased overall levels of Drp1, UV stimulation decreased the levels of cytoplasmic and mitochondrial phospho-Drp1 at Ser637, which contributes to Drp1 activation and recruitment to mitochondria. Furthermore, the Mff-Drp1 interaction increased significantly but no such interaction was observed between Fis1 and Drp1 in response to UV stimulation. Simultaneously, the interaction increased markedly between Fis1 and MiD51/MIEF1 and decreased significantly between MiD51/MIEF1 and Drp1 after UV treatment, which suggests that Fis1 competitively binds to MiD51/MIEF1 to activate Drp1 indirectly. Therefore, Mff and Fis1 directly and indirectly regulate Drp1-mediated mitochondrial fission in response to UV stimulation, respectively. Given that MiD51/MIEF1 and MiD49/MIEF2 are highly conserved across vertebrates (12), the 2 proteins may perform similar activities in some ways. In future studies, it is important to determine whether MiD49/MIEF2 can act like MiD51/MIEF1 in UV irradiation-induced mitochondrial fragmentation.

Wasiak and coworkers (22) demonstrate that Bax stabilizes the association of Drp1 with mitochondrial membranes through sumoylation. Our findings indicated that Mff-Drp1 binding and the Mff-mediated recruitment of Drp1 to the mitochondria did not require Bax during UV stimulation, although we cannot exclude possible contributions from other Bcl2-family proteins. It is more likely that Mff facilitates Drp1 recruitment and Bax promotes a stable membrane-associated form of Drp1 that is involved in later apoptotic events, such as membrane remodeling, which leads to release of cytochrome c and eventual loss of mitochondrial membrane potential (\( \Delta \psi_m \)) (22). Taken together, our data provide evidence of a novel regulatory mechanism of mitochondrial dynamics.

On the basis of our data, we uncovered the machinery involved in mitochondrial dynamics, elucidating the novel role of Mff in regulating Drp1 function and show how the fission proteins are orchestrated to mediate the fission process during UV stimulation (Fig. 6). In brief, Drp1 dephosphorylates at Ser637 and translocates to mitochondria via the membrane-anchored protein Mff after UV irradiation. Mff interacts with accumulated Drp1 on the mitochondrial surface to stimulate Drp1’s GTP-binding activity. Simultaneously, Fis1 promotes mitochondrial fragmentation by competitively binding to MiD51/MIEF1, activating Drp1 indirectly. Therefore, Mff and Fis1 directly and indirectly regulate Drp1-mediated mitochondrial fission in response to UV stimulation, respectively. Given that MiD51/MIEF1 and MiD49/MIEF2 are highly conserved across vertebrates (12), the 2 proteins may perform similar activities in some ways. In future studies, it is important to determine whether MiD49/MIEF2 can act like MiD51/MIEF1 in UV irradiation-induced mitochondrial fragmentation.

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Figure 6. The UV irradiation–induced apoptotic fission pathway. Drp1 dephosphorylates at Ser637 and translocates to mitochondria via membrane-anchored protein Mff. Mff interacts with accumulated Drp1 on the mitochondrial surface to stimulate the former’s GTP-binding activity. Simultaneously, Fis1 promotes mitochondrial fragmentation by competitively binding to MiD51/MIEF1, therefore releasing the suppressive effects of MiD51/MIEF1 on Drp1.

Therefore releasing the suppressive effects of MiD51/MIEF1 on Drp1. Therefore, Mff and Fis1 directly and indirectly regulate Drp1-mediated mitochondrial fission in response to UV stimulation, respectively.

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REFERENCES


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