A uvs-5 Strain Is Deficient for a Mitofusin Gene Homologue, fzo1, Involved in Maintenance of Long Life Span in Neurospora crassa

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Mitochondria are highly dynamic organelles that continuously fuse and divide. To maintain mitochondria, cells establish an equilibrium of fusion and fission events, which are mediated by dynamin-like GTPases. We previously showed that an mus-10 strain, a mutant-sensitive strain of the filamentous fungus Neurospora crassa, is defective in an F-box protein that is essential for the maintenance of mitochondrial DNA (mtDNA), long life span, and mitochondrial morphology. Similarly, a uvs-5 mutant accumulates deletions within its mtDNA, has a shortened life span, and harbors fragmented mitochondria, the latter of which is indicative of an imbalance between mitochondrial fission and fusion. Since the uvs-5 mutation maps very close to the locus of fzo1, encoding a mitofusin homologue thought to mediate mitochondrial outer membrane fusion, we determined the sequence of the fzo1 gene in the uvs-5 mutant. A single amino acid substitution (Q368R) was found in the GTPase domain of the FZO1 protein. Expression of wild-type FZO1 in the uvs-5 strain rescued the mutant phenotypes, while expression of a mutant FZO1 protein did not. Moreover, when knock-in of the Q368R mutation was performed on a wild-type strain, the resulting mutant displayed phenotypes identical to those of the uvs-5 mutant. Therefore, we concluded that the previously unidentified uvs-5 gene is fzo1. Furthermore, we used immunoprecipitation analysis to show that the FZO1 protein interacts with MUS-10, which suggests that these two proteins may function together to maintain mitochondrial morphology.

Throughout their life spans, almost all filamentous fungi continue to grow vegetatively through elongation of their apical tips. The majority of filamentous fungi are thought to grow in this manner for an extremely long time, though the well-studied model fungus Podospora anserina is a notable exception (1). Indeed, hyphal growth of a wild-type strain of the filamentous fungus Neurospora crassa continues for over 2 years, while wild-type P. anserina lives for roughly 20 days (2, 3). This key difference between these closely related fungi is particularly interesting given that they possess the same number of chromosomes, have conserved synteny, and contain genomes composed of similar genes (4).

How do N. crassa and other filamentous fungi maintain such long life spans? Many fungi are multinuclear organisms. Although cell compartments of N. crassa are divided by septa, they are incompletely closed and thus are permeable structures through which organelles such as nuclei and mitochondria can migrate. Furthermore, fungal hyphae can fuse, which results in cell fusion and the existence of multiple organelles in a single cell. These unique features allow cells containing aged and dysfunctional organelles to be replenished with robust organelles, including nuclei containing functional alleles (5).

Other factors that affect fungal life span have been identified through isolation of mutants that exhibit a senescence phenotype. In N. crassa, mutation of some nucleus-expressed genes results in a shortened life span. The natural death (nd) mutant was obtained through UV-induced mutagenesis and showed cessation of growth in successive transfers under all nutritional conditions (6). The senescent (sen) mutant was first identified as a short-lived mutant of Neurospora intermedia; the sen mutant of N. crassa was obtained by introgression of two species (7). Both the nd and sen strains accumulate deletions of mitochondrial DNA (mtDNA), though it is speculated that these arise through distinct mechanisms (8–10). Three UV- or mutagen-sensitive mutants of N. crassa, i.e., the uvs-4, uvs-5, and mus-10 mutants, also show a senescence phenotype accompanied by an accumulation of mtDNA deletions (11–14).

The mus-10 mutant was originally isolated as a strain that showed high sensitivity to methyl methanesulfonate (MMS) (15). Because of this mutagen sensitivity, the protein encoded by mus-10 was thought to have a function in the DNA repair process, though its precise function could not be determined. Moreover, although mitochondria of wild-type N. crassa are long and tubular, those of the mus-10 strain are fragmented, even in relatively young cultures. Using a gene rescue approach, we found that the mus-10 gene encodes a protein that has an F-box domain. The physical interaction between MUS-10 and SCON-3 (an Skp1 homologue) and the necessity of the F-box domain for MUS-10 function suggested that MUS-10 is part of an SCF E3 ubiquitin ligase complex and functions in the degradation of an unknown target protein(s) (12). Thus, MUS-10 may act in a ubiquitin-proteasome pathway to regulate turnover of proteins required for maintenance of mitochondria and the life span (12).

Mitochondria undergo repeated fusion and fission events that are regulated primarily by dynamin-like GTPases (16, 17). Mitofusins (MFN1 and MFN2) in mammals (18) and Fzo1 in Saccharomyces cerevisiae (19, 20) are conserved transmembrane, dy-
namin-related GTPases that are exposed to the cytosol and function in the fusion of the mitochondrial outer membrane. Other dynamin-like GTPases, such as OPA1 in mammals (21, 22) and Mgm1 in yeast (23), are involved in fusion of the mitochondrial inner membrane, while DRP1 (24) and its yeast homologue Dnm1 (25) play a role in mitochondrial fission.

It is known that abnormal mitochondrial morphology is associated with genetic diseases and neural degeneration. For example, mutation of MFN2 causes Charcot-Marie-Tooth neuropathy type 2A (CMT2A), while OPA1 deficiency results in autosomal dominant optic atrophy (21, 26). In budding yeast, these genes are required for normal growth, respiration, and mtDNA inheritance (20, 27). In N. crassa, the GTPases that regulate mitochondrial morphology are conserved but have not been characterized in great detail (28).

Recently, the relationship between mitochondrial dynamics and aging has received considerable attention. Although Young P. anserina and S. cerevisiae cells harbor long filamentous mitochondria, the percentage of cells that have fragmented (abnormal) mitochondria increases with age (29). Moreover, for P. anserina and S. cerevisiae, dnm1 mutants that are mitochondrial fission deficient have an extended life span. Conversely, overexpression of PaDNM1 in P. anserina shortens the life span (30). In yeast, Δmgl1 and Δfzo1 mutants, which exhibit abnormal mitochondrial fission, have a shortened life span (31, 32). Taken together, these findings suggest that aging is associated with mitochondrial fragmentation. However, contradictory results have also been reported. In PaFZO1-overexpressing P. anserina, mitochondrial fragmentation is not observed, but the life span remains comparable to that of the wild type (30). In nematodes, DRP1 mutants do not exhibit an abnormal life span (33). Thus, it remains unclear whether mitochondrial fragmentation is related to aging. Since being deficient for mitochondrial fusion is usually lethal, it is difficult to study mitochondrial fragmentation. This lethality likely results from the compromised respiration of fragmented mitochondria, as this respiration is the primary source of ATP generation for most organisms (34).

In this study, we reveal that the uvs-5 mutant exhibits phenotypes that resemble those of the mus-10 strain: sensitivity to MMS and UV light, premature senescence, and abnormally fragmented mitochondria. Furthermore, using gene rescue and gene knock-in strategies along with sequence analysis, we confirm that the uvs-5 mutant is deficient for the mitofusin-encoding fzo1 gene. Finally, we show that the UVS-S/FZO1 protein physically interacts with the F-box protein MUS-10, which suggests that turnover of FZO1 is regulated by the SCF–MUS-10 complex.

**MATERIALS AND METHODS**

**Strains, plasmids, and genetic manipulations in N. crassa.** The N. crassa strains used in this study are listed in Table 1. Growth and handling of N. crassa were performed as previously described (37). Transformation was carried out according to the method of Ninomiya et al. (36). A bialaphos resistance gene (Bar) whose expression is controlled by the Aspergillus nidulans trpC gene promoter was used as a selectable marker in transformations of N. crassa. This marker, originating from pBARGEM-7 (38), was utilized for this study by placing EcoRV restriction sites at each end of the PCR-amplified DNA marker and subsequently cloning it into pGEM-T Easy (Promega) to produce pGEM-Bar.

**Age matching.** Since the uvs-5 and mus-10 strains display senescence phenotypes, their age in culture may affect their remaining life span and mutagen sensitivities. Thus, proper analysis of phenotypes requires age matching of conidia. To accomplish this, the mus-10 and uvs-5 mutants were backcrossed to a wild-type strain, C1-T10-28a or C1-T10-37A, and the resulting ascospores were randomly isolated and used as stocks after 7 days of culture on solid medium. These age-matched progeny were further characterized as wild-type, mus-10, and uvs-5 strains based on their sensitivities to UV light and MMS. Stocks of conidia with the appropriate phenotypes were confirmed by sequence analysis and then used in further experiments (12).

**Measurements of linear growth rate and life span.** Apical growth of hyphae was measured in race tubes as previously described (39). Briefly, race tubes containing Vogel’s minimal agar medium with 1.2% sucrose were inoculated with conidia at one end of the tube and incubated at 25°C. To determine the apical growth rates of the various strains, hyphal tip extension was recorded once or twice a day. When hyphae traversed the tube, a small amount of mycelia was transferred to a fresh tube, and the entire process was repeated. Strains that ceased growth at some point in a race tube were deemed to have reached the end of their life span.

**Quantitative analysis of mutagen sensitivity.** The mutagen sensitivities of N. crassa strains were investigated through spot tests as previously described (12). Conidia were suspended and adjusted to a concentration of 1 × 10⁶ conidia/ml. These suspensions were serially diluted five times (1:4), and then 10-µl aliquots of each conidial suspension were transferred to agar plates containing minimal medium and 1% sorbose to

| TABLE 1 N. crassa strains used in this study |
|------------------------------------------------|
| **Strain** | **Genotype** | **Origin, source, or reference** |
| Wild type and original uvs-5 mutants |
| C1-T10-28a | A | 35 |
| C1-T10-37A | A | 35 |
| 74-OR23-1A | A | FGSC |
| FGSC2970 | a uvs-5 | FGSC |
| FGSC2971 | a uvs-5 | FGSC |
| Age-matched strains |
| WT-bc-1 | Undetermined | This study |
| u5-bc1WT-bc-1 | a uvs-5 | This study |
| KTO-10H-10A | A mus-10::hph | 12 |
| Strains for fzo1 knockout |
| FGSC9719 | a mus-52::Bar | FGSC |
| fzo1het | a mus-52::Bar + mus-52::Bar | This study |
| fzo1::hph | fzo1::hph | This study |
| Strains for FLAG-tagged FZO1 expression |
| FGSC6103 | A his-3 | FGSC |
| u5his3-29 | uvs-5 his-3 | This study |
| u5-FZO1 | uvs-5 his-3 fzo1::FLAG | This study |
| u5-u5FZO1-11 | uvs-5 his-3 fzo1Q368R::FLAG | This study |
| Strains for knocking in fzo1 |
| 54yo-828-4 | a mus-52::hph | 36 |
| u5zobbar-1322 | a mus-52::hph fzo1Q368R::Bar | This study |
| WTfobar-4 | a mus-52::hph fzo1Q368R::Bar | This study |
| u5zobbar-1322-6 | fzo1Q368R::Bar | This study |
| WTfobar-4-4 | fzo1Q368R::Bar | This study |
| Strains for HA-tagged MUS-10 expression |
| KRA-m10his3-5 | mus-10::hph his-3 | 12 |
| M10HA | mus-10::hph his-3 | This study |
| hsp10::mus-10-HA | This study |
TABLE 2 Primers used for sequencing of fzo1

| Primer       | Sequence (5′ to 3′)                     |
|--------------|----------------------------------------|
| fzo-2kl      | TGGTACCTTGACTGCTGCTCATAT              |
| ncu00436-1kl | AAAGAAGGGCGGCTAGATGCTG                |
| KK09-0       | TCTAGAACATGACCAGAGAGTTACTACCCGT       |
| fzo-1-560    | ACTGGCAATGCTTCAAGCAGCTG               |
| fzo-1-1130   | TATACCAGGCTCTCTGCTGACAG              |
| fzo-1-1460   | CACTTCGTCGACATCTTCTGGG               |
| fzo-1-1690   | TACTCCTCAACACATTTGCAGACG             |
| fzo-2300     | GTCGTCGGTACATGGTCTGTT               |
| fzo-20r      | GAGGTTAAGCGGATCTGTTAGG              |
| fzo-430r     | GTCTGGTTCATCGTCTGAGCTTT            |
| fzo-1360r    | GCAACGCTCTCTATATCTCTAA            |
| KK10-2800r   | GGATCCCGTGGAACACCTCCAATACCAAGCA    |
| ncu00436-1kR | GCCCTCTACATCCGTCGCAGAT          |

produce colonial growth of N. crassa. MMS (0.015%) or histidine (50 μg/ml) was added to the growth medium when necessary. To analyze UV sensitivity, conidial suspensions were spotted onto agar plates as described above, dried, and then irradiated with UV light at a dose of 300 J/m². The plates were placed in a 30°C incubator for 2 days, after which photographs were taken to document the amount of growth.

**Sequence analysis.** To determine the DNA sequence of NCU00436.5 (fzo1) in the uvs-5 mutant, the fzo1 open reading frame (ORF) and flanking regions, from position −778 bp relative to the translational start ATG to position +3818, were amplified using PCR and cloned into pGEM-T Easy. Sequencing reactions were carried out using primers that were designed to anneal at about 600-nucleotide (nt) intervals (Table 2), as well as a BigDye sequencing kit (Applied Biosystems). Sequencing samples were run on an Applied Biosystems 3130 genetic analyzer. The obtained sequences were compared with those from the Neurospora database (40).

**Examination of mitochondrial morphology.** Mitochondria were observed as described by Kato et al. (12), with slight modifications. Conidia were inoculated on Vogel’s minimal plates containing 1.2% sucrose and 2% agar and incubated overnight at 30°C. MitoTracker Green FM (1 μM; Invitrogen) was then dropped onto the mycelial growth front. After 20 min at room temperature, a piece of mycelium–containing medium was transfected to a glass slide by use of a spatula and then covered with a glass coverslip. Differential interference images of hyphae and mitochondria stained by MitoTracker Green FM were captured using a confocal laser scanning microscope (FV-1000D; Olympus) at an excitation wavelength of 473 nm.

**Generation of FLAG-tagged wild-type and mutated FZO1.** DNA encoding the FLAG-tagged FZO1 protein was specifically integrated into the his-3 locus as described by Freitag et al. and Kato et al. (12, 41). Primers KK09-ncu00436c-5 (5′-TCTAGAACATGGACAGATCTACCCG T′-3′) and KK10-ncu00436c-3 (5′-GGATCTCCTGGAACCTCCACTAC CAGCA-3′) were used to amplify the full-length fzo1 gene (3,084 bp), using genomic DNA from wild-type N. crassa (74-OR23-1A) as the template. This DNA fragment was digested with XbaI and BamHI and then cloned into the corresponding sites of pFLAG (12). The resulting construct, named pFZOFLAGC, could express FLAG-tagged wild-type FZO1 and was digested with XbaI and Smal restriction sites, respectively, to the ends of the mus-10 ORF. The PCR product was digested with XbaI and Smal and inserted into the corresponding sites of pHAC1. Finally, this construct was introduced into an mus-10 his-3 double mutant by electroporation, and an HA-tagged MUS-10-expressing strain, M10HA, was isolated.

**Immunoprecipitation and Western blot analysis.** After overnight incubation of 10° conidia in 20 ml liquid Vogel’s minimal medium at 30°C, cells were collected and homogenized using a MicroSmash MS-100 homogenizer (Tomy) at 3,500 rpm for 3 min at 4°C in extraction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10% glycerol, 0.3% Triton X-100) containing Complete protease inhibitor cocktail (EDTA-free; Roche). The extracts were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatants were gently mixed with anti-FLAG M2 affinity gel (Sigma) for 1 h at 4°C and washed five times with extraction buffer. Protein was eluted using 4× SDS sample buffer (8% SDS, 40% glycerol, 250 mM Tris-HCl, pH 6.8, 0.08% bromophenol blue [BPBr]), boiled, and electrohoresed through 10% polyacrylamide gels. Proteins from the gels were transferred to polyvinylidene difluoride (PVDF) membranes, which were then probed with mouse anti-FLAG M2 monoclonal antibody (Sigma) or anti-HA monoclonal antibody (HA-11; Covance) for detection of tagged proteins as previously described (12).

**Construction of fzo1Q368R strain.** To construct a strain carrying fzo1 encoding a Q368R mutation in its product (fzo1Q368R strain), a fragment including part of the fzo1ORF (bp 1 to 1360) and a 2,380-bp region upstream of the start codon was amplified by a PCR using genomic DNA from the uvs-5 mutant and the primers fzo-2kl (5′-TGGTACCTTGACTGCTGCTCATAT-3′) and fzo1500r (5′-GCAACGCTCTCTATATCTCTAA-3′). After cloning of the PCR product into pGEM-T Easy (Promega), the EcoRI fragment from pGEM-Bar, containing the Bar gene, was inserted into the EcoRI site located upstream of the fzo1 ORF (bp −360). The resulting construct was introduced into a mus-52::hph strain that is deficient for nonhomologous end joining and thus facilitates gene integration at endogenous loci (36). Knock-in transformants among the bialaphos-resistant strains were confirmed by a PCR using primers located in the region upstream of the fzo1 ORF and flanking the Bar gene (see Fig. 5A). The presence of an ∼1,900-bp band (and absence of a ∼500-bp band indicative of no Bar insert) confirmed that the transmigrant was homokaryotic and that integration had not occurred at an ectopic site. DNA fragments containing nt 1103 of the fzo1 gene were amplified using a pair of primers, one of which was inside the fzo1 gene and the other of which was complementary to the genomic sequence outside the introduced fragment. The presence or absence of the sequence encoding the A1103G base substitution in the fzo1 gene was confirmed by DNA sequencing of the amplified DNA fragment.

**RESULTS**

The uvs-5 and mus-10 mutants have similar phenotypes. There are several similarities between the uvs-5 and mus-10 mutants, including sensitivity to UV light, instability of mtDNA, and a se-nescence phenotype (11, 13, 14, 44, 45). To compare the life spans of the two strains, conidia from age-matched uvs-5 and mus-10 mutants were inoculated into race tubes. Both mutants stopped growing at almost the same elapsed time (for the uvs-5 strain, phenotypes was confirmed using Western blot analysis with the monoclonal anti-FLAG M2 antibody (F3165; Sigma) as previously described (42, 43).

**Generation of HA-tagged MUS-10.** To create a MUS-10 protein with a hemagglutinin (HA) epitope tag at its C terminus, we first added the sequence encoding the HA tag to the his-3 targeting vector pMF272 (41). This was achieved through PCR amplification of a DNA fragment that contained linker sequences and the HA epitope tag, which was then digested with Smal and EcoRI and inserted into the Smal/EcoRI sites of pMF272 to produce plasmid pHAC1. The full-length mus-10 ORF was PCR amplified using a CDNA template and the primers m10-6 (5′-TCTAGAATTACGTCGTCCTCCTCTTCA-3′) and m10-7 (5′-TCCGGGT GTGTCGGGTGATCGGTCT-3′), which added Xbal and Smal restriction sites, respectively, to the ends of the mus-10 ORF. The PCR product was digested with XbaI and Smal and inserted into the corresponding sites of pHAC1. Finally, this construct was introduced into an mus-10 his-3 double mutant by electroporation, and an HA-tagged MUS-10-expressing strain, M10HA, was isolated.
and for the mus-10 strain, 416.6 ± 10.2 h (n = 5) for the mus-10 strain. The maximum length of growth was 87.6 ± 1.1 cm (n = 5) for the mus-10 strain and 89.1 ± 0.5 cm (n = 5) for the uvs-5 strain. All data are shown in Fig. S1 in the supplemental material. (B) Spot test analysis was performed using age-matched conidia as described for panel A, along with the original FGSC uvs-5 mutant (FGSC uvs-5; strain FGSC2970) and the seventh subculture of the mus-10 strain (strain KTO-10H-10A). Ten-microliter conidial suspensions (1 × 10^6 conidia/ml or one of five 1:4 serial dilutions) were spotted onto agar plates containing 0.01% MMS or 0.5 mg/ml histidine, as indicated. In the case of UV, conidia were exposed to 300 J/m² of UV after spotting. Plates were photographed after incubation for 2 days at 30°C.

417.6 ± 14.6 h [n = 5]; and for the mus-10 strain, 416.6 ± 10.2 h [n = 6]) (Fig. 1A; see Fig. S1 in the supplemental material). This result reveals that the life span of the uvs-5 mutant is similar to that of the mus-10 mutant.

Next, we examined the mutagen sensitivities of the two mutants. Käfer and our group previously showed that the mus-10 mutant is sensitive to MMS but not to histidine (12, 46). On the other hand, the uvs-5 mutant was reported to be MMS insensitive (47), and its growth was retarded in medium supplemented with histidine (14). To confirm the mutagen sensitivities of these strains, we analyzed their growth through spot tests using age-matched strains. Although identical amounts of conidia were used for these experiments, growth of the uvs-5 strain in the absence of mutagens was hindered compared to that of the mus-10 and wild-type strains, indicating a decreased viability. In contrast to previously published reports, our results indicate that the spectrum of mutagen sensitivities of our uvs-5 mutant is the same as that of an age-matched mus-10 mutant: a high sensitivity to both UV and MMS and insensitivity to histidine (Fig. 1B). Interestingly, the uvs-5 strain from the FGSC and the aged mus-10 strain were sensitive to MMS, UV, and histidine, which suggests that the uvs-5 strain from the FGSC is an older strain and that histidine sensitivity is an aging-related phenomenon in some strains. Similarly, the aged mus-10 strain displayed sensitivity to hydroxyurea (HU) and 4-nitroquinoline 1-oxide (4NQO), while a young mus-10 strain did not (data not shown). The aged nature of the uvs-5 strain from the FGSC is further supported by the fragmented mitochondria that it harbors (Fig. 2).

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Normal (wild-type) mitochondria show a tubular morphology that is thought to result from a dynamic equilibrium of mitochondrial fusion and fission (Fig. 2). Our previous studies have shown that the mus-10 mutant possesses fragmented mitochondria, even in earlier subcultures, in which its hyphae grow normally (Fig. 2) (12). Since the uvs-5 mutant exhibited phenotypes similar to those of the mus-10 mutant with regard to life span and mutagen sensitivity, we hypothesized that the uvs-5 mutant may also have altered mitochondria. To test this hypothesis, hyphae of the uvs-5 mutant were stained with a mitochondrion-specific dye, MitoTracker Green FM, and
then visualized under a fluorescence microscope. Earlier subcultures of the uvs-5 mutant contained tubular mitochondria, as observed in wild-type strains (Fig. 2). However, by the 5th subculture, uvs-5 mitochondria were fragmented similarly to those observed in hyphae of the mus-10 strain. It has also been reported that the uvs-5 strain is sterile in homozygous crosses, while the mus-10 strain is not (15, 48).

Since our mutagen-sensitivity profile of the uvs-5 strain differed from previously published reports, we wanted to determine whether a barren phenotype was observed for a homozygous cross of the two uvs-5 strains used in this study. Both uvs-5 strains (our isolate and the strain acquired from the FGSC) could not form protoperithecia and thus were deemed female sterile (data not shown).

**Analysis of the fzo1 gene in the uvs-5 mutant.** The uvs-5 gene was genetically mapped to the right arm of linkage group III and was shown to be linked to the vel mutation (14). The vel mutation was mapped between phe-2 and tyr-1 (49, 50) (Fig. 3A). The phe-2 and tyr-1 genes have been assigned locus numbers NCU00409.5 and NCU00468.5, respectively, by the Neurospora genome project. Using the *N. crassa* genome database, 56 putative ORFs were found between NCU00409.5 (phe-2) and NCU00468.5 (tyr-1) (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html). We noted that a mitofusin (yeast FZO1) gene homologue, NCU00436.5, was among these ORFs (Fig. 3A). The NCU00436.5 ORF encodes a protein that shares 32.6% identity and 46.3% similarity to budding yeast Fzo1 (Fig. 3B). The predicted GTPase and transmembrane domains are conserved between Fzo1 from budding yeast and its *N. crassa* homologue (Fig. 3C). We therefore designated NCU00436.5 the fzo1 gene of the *uvs*-5 strain. This mutation changes the amino acid at position 368 from glutamine (Q) to arginine (R).

**FIG 3** *N. crassa fzo1* is a mitofusin gene homologue. (A) Genetic and physical map of the region between tyr-1 and phe-2. On the right arm of linkage group III (LGIIIIR), 56 putative ORFs are presented. (B) Structures of mitofusin homologues. The lengths of the polypeptides are shown on the right. GTPase and transmembrane domains are indicated by black and gray boxes, respectively. (C) Alignment of amino acid sequences of the GTPase domains of mitofusin homologues. Identical and similar residues among proteins are represented by black and gray boxes, respectively. An asterisk shows the glutamine residue that is changed to arginine in the *uvs*-5 mutant. The conserved GTPase motifs, G1, G2, G3, and G4, are outlined. (D) Single base substitution detected in the fzo1 gene of the *uvs*-5 strain. This mutation changes the amino acid at position 368 from glutamine (Q) to arginine (R).
mitochondrial morphology observed in older subcultures of the uvs-5 mutant was caused by a deficiency in fzo1.

To determine whether the fzo1 gene in the uvs-5 mutant harbored any mutation(s), the fzo1 ORF was cloned from the uvs-5 mutant, and its sequence was determined. A single A-to-G substitution was found at nt 1,103. This mutation causes a single amino acid substitution, from glutamine (Q) to arginine (R), at amino acid 368 of the polypeptide, which is located in the GTPase domain of FZO1 (Fig. 3D).

**Confirmation that uvs-5 is fzo1.** To determine whether the mutation we identified within the fzo1 gene of the uvs-5 mutant was responsible for the resulting phenotypes, we first employed a gene rescue approach. The FLAG-tagged FZO1 protein was expressed in the uvs-5 mutant under the control of the ceg-1promoter. Expression of FZO1 was verified by Western blotting using an anti-FLAG monoclonal antibody (Fig. 4A). Germinating conidia from the host strain, the uvs-5 his-3 strain, contained tubular mitochondria similar to those of the wild-type strain and early subcultures of the uvs-5 strain, which verified that the conidia used for the transformation were young (Fig. 2 and 4B, uvs-5 strain). As shown in Fig. 4B, the vector-control transformant (uvs-5-vector strain) showed fragmented mitochondria resembling those of the aged uvs-5 strain (Fig. 2D and 4B). The uvs-5-vector strain senesced slightly faster than the uvs-5 strain, but this was not surprising because the former strain had aged throughout the transformation process (Fig. 1A and 4C). Age matching of the uvs-5-vector strain was not practical, as this strain cannot be backcrossed to the wild type because it contains 2 copies of the fzo1 ORF, which can lead to mutation via repeat-induced point mutation during the sexual phase. The transformant expressing exogenous wild-type FZO1 maintained tubular mitochondria after the transformation procedure, suggesting that the uvs-5 mutation had been rescued [Fig. 4B, uvs-5+FZO1(WT) strain]. Furthermore, expression of FZO1 also rescued the senescence phenotype of the uvs-5 strain (Fig. 4C) and partially restored resistance to MMS, though this transformant was still slightly more sensitive to MMS than the wild-type strain (Fig. 4D). However, a slight increase in MMS sensitivity was also observed in wild-type strains expressing ectopic FZO1, suggesting that overexpression and/or improper regulation of fzo1 may induce drug sensitivities (data not shown).

To demonstrate further that the uvs-5 phenotypes resulted from FZO1 deficiency, a mutant FZO1 protein was expressed in a uvs-5 background [uvs-5+FZO1(Q368R) strain]. This mutated version of FZO1, FZO1(Q368R), carried the same amino acid substitution we observed in the uvs-5 mutant. Interestingly, although expression of FZO1(Q368R) was driven by the ceg-1 promoter, which was also used to express wild-type FZO1 in our previous experiments, the protein level of the mutant protein was much lower than that of the wild-type version (Fig. 4E). Furthermore, introduction of the mutant FZO1 protein could not complement the mutagen sensitivities and fragmented mitochondria of the uvs-5 strain (Fig. 4B and D). While the apical growth of the uvs-5+FZO1(Q368R) strain continued for slightly longer than that of the control strain (uvs-5+vector), this mutant still displayed a senescence phenotype (Fig. 4C).

Since overexpression of mitofusins or various FZOs can rescue mitochondrial fragmentation and other related phenotypes caused by mutations of other genes (53, 54), it was possible that expression of wild-type FZO1 in the uvs-5 background complemented the uvs-5 mutant phenotypes but that fzo1 and uvs-5 were not allelic. To determine if this was the case, we employed a knock-in strategy to replace the fzo1 gene in a wild-type strain with a mutated version. The fzo1 ORF and flanking upstream region were amplified using genomic DNA of the uvs-5 mutant as a template and then cloned. A bialaphos resistance gene (Bar) was then inserted into the EcoRI restriction site located in the region upstream of the fzo1 ORF (Fig. 5A). This construct was introduced into the mus-52::hph strain to facilitate targeted integration into the desired locus (36). Since ectopic integration of foreign DNA is strongly reduced in this host strain, the construct should have integrated into the endogenous fzo1 gene via homologous recombination. More than 100 bialaphos-resistant transformants were obtained by electroporation, and three homokaryotic strains were isolated (Fig. 5B and data not shown). For one of these homokaryons, it was confirmed through sequence analysis that the fzo1 gene was replaced with the mutant construct [fzo1Q368R::Bar]; the single colon indicates the artificial insertion of the marker gene into the flanking region of fzo1, whereas double colons in other designations indicate that the gene was replaced by a marker gene, and thus disrupted). However, sequence analysis showed that the other two isolates maintained the wild-type fzo1 ORF despite integration of the Bar gene into the region upstream of fzo1 [fzo1WT::Bar]. The fzo1Q368R::Bar strain showed sensitivities to UV and MMS that were similar to those of the uvs-5 mutant (Fig. 5C). These phenotypes were caused by knock-in of the Q368R mutation, not due to insertion of the Bar gene into the fzo1 upstream region, as the fzo1WT::Bar strain did not show altered mutagen sensitivities (Fig. 5C).

If the uvs-5 and fzo1Q368R mutations were at different loci, then a heterokaryon containing nuclei from both mutants should show evidence of complementation. To examine this hypothesis, heterokaryons that consisted of nuclei originating from two distinct strains were formed and their sensitivities to various mutagens examined. Indeed, a heterokaryon formed from the combination of the fzo1Q368R::Bar strain and the uvs-5 strain showed the same mutagen sensitivities as the original strains (Fig. 5D). As a control, we also examined whether nuclei from the mus-10 strain could complement the deficiencies of the uvs-5 strain by using a heterokaryon. Since this heterokaryon was not sensitive to MMS and UV, the mus-10 and uvs-5 mutations were confirmed as nonallelic mutations (Fig. 5D). These results strongly suggest that fzo1 and uvs-5 are allelic and that the A1103G substitution is responsible for the phenotypes exhibited by the uvs-5 mutant.

It should be noted that the fzo1Q368R::Bar strain senesced after about 100 h of growth (data not shown) and that its mitochondria were fragmented in the 1st subculture (Fig. 5E). These phenotypes remained in progeny that were isolated after backcrossing to a wild-type strain and removal of the mus-52 mutation. The slight differences between the original uvs-5 mutant and our knock-in strain are likely explained by variations in their genetic backgrounds.

**Fzo1 is essential in N. crassa.** We attempted to generate a disruption mutant of fzo1 by using a gene replacement procedure. The wild-type fzo1 ORF and flanking upstream region were amplified by PCR and cloned. Part of the ORF, including the sequence encoding the GTPase domain, was then replaced with a hygromycin resistance gene (hph). This construct was introduced into macroconidia from the mus-52::Bar strain by electroporation, which facilitates targeted replacement of the desired gene.
Over 100 hygromycin-resistant transformants were obtained. Since macroconidia usually contain 2 or more nuclei, each of these transformants could be heterokaryotic, possessing an hph-disrupted fzo1 gene in one nucleus and a wild-type fzo1 gene in another. In an attempt to isolate a homokaryon with the disrupted fzo1 gene, repeated single-colony isolation or backcrossing to a wild-type strain was performed. To check whether resulting strains were homokaryotic, we performed PCR analysis (see Fig. S2 in the supplemental material). Despite our best efforts, we were unable to isolate homokaryotic fzo1 knockouts. This suggests that the fzo1 gene is essential for cell viability, as is the case in higher eukaryotic cells.

**Interaction of MUS-10 and FZO1.** Since the mus-10 and fzo1 strains exhibited similar phenotypes, it was possible that MUS-10, which is part of an E3 ubiquitin ligase that regulates protein turnover, was able to bind to and regulate FZO1. To investigate this...
possibility, we examined whether the two proteins interacted physically through immunoprecipitation experiments. HA-tagged MUS-10 and FLAG-tagged FZO1 were expressed under the control of the constitutive cpg-1 promoter in separate strains. Whole-cell extracts of each strain were mixed and immunoprecipitated with anti-FLAG antibody. We then detected the MUS-10 protein by Western blotting using an anti-HA antibody. HA-tagged MUS-10 was immunoprecipitated only when it was first mixed with the FLAG-tagged FZO1 protein (Fig. 6). This result indicates that MUS-10 interacts directly or indirectly with FZO1.

DISCUSSION

In this study, we show that (i) the uvs-5 strain harbors a point mutation in the fzo1 gene, an N. crassa mitofusin gene homologue; (ii) the phenotypes of the uvs-5 strain are complemented by expression of wild-type but not mutated FZO1; (iii) the fzo1 mutation present in

FIG 5 Knock-in of the A1103G mutation into the fzo1 gene. (A) A construct carrying the A1103G mutation of fzo1 and the Bar marker was used for the knock-in procedure. The numbers indicate distances (base pairs) from the start codon of fzo1, while the asterisk marks the location of the A1103G mutation. Arrows indicate the positions of PCR primers used to amplify the DNA fragments used for sequence analysis. (B) Confirmation of Bar gene insertion into the upstream region of the fzo1 gene. The products of PCR amplification using a wild-type genome template and the primers shown in panel A were approximately 500 bp long. When the Bar gene was inserted, the size of the PCR product increased to roughly 1,900 bp. Construct, plasmid used for knock-in procedure. (C) Two bialaphos-resistant homokaryotic transformants from the knock-in procedure described for panel A, i.e., the fzo1Q368R:Bar and fzo1WT:Bar transformants, possess a mutant (A1103G) and a wild-type copy of fzo1, respectively. The mus-52 gene in these strains has been disrupted with a hygromycin resistance cassette, which leads to efficient gene targeting. Mutagen sensitivities were tested by spot test analysis as mentioned in the legend to Fig. 1. (D) Complementation experiments. Conidial suspensions of the uvs-5, fzo1Q368R:Bar, and mus-10 strains were spotted onto solid medium as described in the legend to Fig. 1. Suspensions containing mixtures of conidia from the uvs-5 and mus-10 strains (uvs-5 + mus-10), the fzo1Q368R:Bar and mus-10 strains (fzo1Q368R:Bar + mus-10), or the uvs-5 and fzo1Q368R:Bar strains (uvs-5 + fzo1Q368R:Bar) were also spotted onto plates. For these experiments, suspensions containing a total of 10^3, 10^4, or 10^5 conidia (from left to right) were used. (E) Mitochondrial morphologies of hyphae derived from the first subcultures of homokaryotic conidia from the fzo1Q368R:Bar and fzo1WT:Bar strains. The procedure is outlined in the legend to Fig. 2.

FIG 6 Coimmunoprecipitation assays of FLAG-tagged FZO1 and HA-tagged MUS-10. Immunoprecipitation assays were performed in the presence (+) or absence (−) of whole-cell extracts isolated from strains expressing FLAG-tagged FZO1 (FZO1-FLAG) or HA-tagged MUS-10 (MUS-10-HA). When tagged proteins were absent (−), whole-cell extracts from the parental strain (his-3) were used instead. Immunoprecipitation was carried out using anti-FLAG antibodies. Input and immunoprecipitated (IP) samples were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA antibodies.
the *uvs-5* strain causes hypersensitivities to UV and MMS, as well as a shortened life span; and (iv) the *uvs-5* and *fzo1* mutants cannot complement each other. Taken together, these results strongly suggest that *uvs-5* and *fzo1* are allelic.

**Mitofusins are essential for life.** An *N. crassa* gene knockout project has been under way since 2005, and as a result, thousands of knockout strains have been deposited at the FGSC. A mutant with a knockout of NCU00436.5 (*fzo1*), strain FGSC17478, is publicly available, but unfortunately, our experiments show that this strain is a heterokaryon containing both wild-type and mutant *fzo1* (data not shown). Our attempts to obtain an *N. crassa fzo1* knockout strain were also unsuccessful, which strongly suggests that the *fzo1* gene is essential for viability of *N. crassa*. Conversely, deletion of *FZO1* in budding yeast is not lethal but does lead to mtDNA loss, a respiration deficiency, an inability to grow on media containing a nonfermentable carbon source, and a shortened life span (19, 20, 31). However, mitofusins are essential for viability in almost all other eukaryotes. Two mitofusin homologues, Fzo and Marf, exist in *Drosophila*. In contrast to Fzo, Marf is a ubiquituous mitofusin, and its knockdown leads to lethality (52). Mammals also have two *fzo1* homologues, MFN1 and MFN2. Deletion of either of these mitofusin genes results in embryonic lethality (51). This is also true for two other mitochondrial fusion factors, Ugo1 and Opa1 (23, 55, 56). Homologues of *UGO1* and *OPA1* are also found in *N. crassa* and are annotated NCU07793.5 and NCU01255.5, respectively. Knockout mutants of these genes are also available from the FGSC, namely, strains FGSC18672 and FGSC17555, respectively, though they are maintained as heterokaryons, indicating that these genes are essential for viability. Thus, mitochondrial fusion is essential for viability in *N. crassa*, as is the case in higher eukaryotes. The *N. crassa uvs-5* mutant will be a useful model for studying the relationship between mitochondrial fusion and early senescence in higher eukaryotes.

**Characteristics of the *uvs-5* mutant.** In this study, we show that the *uvs-5* mutation confers an imbalance of mitochondrial fission and fusion, as well as mutagen sensitivities. It is possible that these phenotypes are correlated with the stability of the FZO1 protein. When FZO1 proteins were expressed in *N. crassa* by use of the *ccc-1* promoter, the protein level of the FZO1 mutant was much lower than that of wild-type FZO1 (Fig. 4). The cause of this discrepancy is unclear. However, these results reveal the possibility that the Q368R mutation may alter the stability of the FZO1 protein, which then leads to an imbalance of mitochondrial fusion and fission. It should also be mentioned that although we have confirmed that the Q368R mutation affects FZO1 function, we have not specifically examined the GTPase activity of the mutant protein.

Conidia from the first subcultures of age-matched *uvs-5* and *mus-10* mutants display similar spectra of mutagen sensitivity, including an increased sensitivity to histidine in aged strains. Furthermore, the accumulation of deletions in mtDNA in both strains also occurs in an age-related manner (11, 12). While the onset of mitochondrial fragmentation differs between our *uvs-5* and *fso1* strains, it is clear that the *mus-10* mutant, *uvs-5/fso1* mutants display an imbalance of mitochondrial dynamics. Regulation of mitochondrial morphology is very important to the maintenance of mitochondrial function. For example, tubular and net-like mitochondrial networks are able to produce ATP more efficiently than small and fragmented mitochondria (34). In aged *mus-10* and *uvs-5* mutants, the fragmented mitochondria may not be able to produce enough ATP to maintain essential functions, including DNA repair, and this deficit would subsequently lead to further deficiencies. It is still unclear whether ATP productivity is correlated with the repair of damaged DNA. However, it is interesting that a juvenile *uvs-5* strain that has normal mtDNA and a tubular mitochondrial morphology is still hypersensitive to UV and MMS (see below).

**Physiological significance of mitofusins.** Since mitofusins have an important role in the fusion of mitochondria, mitochondrial fragmentation may be induced in *N. crassa* by a defect in *fzo1* (Fig. 2). Dysfunctional mitochondrial dynamics are associated with various neurodegenerative diseases, including Parkinson’s disease (57). It is thought that the molecular functions of fusion and fission may govern mitochondrial homeostasis (58, 59). The mitochondrial defects caused by abnormal mtDNA or mitochondrial proteins, some of which result from exposure to reactive oxygen species that are prevalent in mitochondria, are complemented through fusion with normal mitochondria. On the other hand, mitochondrial fission acts to separate aberrant mitochondria, which are then degraded through autophagy (60). Mitochondrial fusion and fission are also thought to function in mitochondrial biogenesis and the cellular distribution of energy (61). Therefore, characterization of factors involved in mitochondrial fusion and fission is very important for uncovering the relationship between mitochondria and cell maintenance (60, 61).

In our experiments, the *uvs-5* mutant showed female sterility. However, it has been reported that formation of protoperithecia in the *uvs-5* mutant is normal (48). It is not clear why such a discrepancy exists. Our results suggest that the *uvs-5* mutant harbors some kind of deficiency in gametogenesis. Similarly, in the spermatogenesis of *Drosophila*, mitochondria form a special aggregated structure called a nebkern, whose formation depends on the function of Fzo (62). *Drosophila* Fzo is a spermatogenesis-specific mitofusin, and its deficiency leads to male sterility. These characteristics imply that mitochondrial fusion by mitofusins can be important for sexual reproduction.

**Ubiquitination of mitofusins.** In this study, we determined that the *mus-10* and *uvs-5* strains exhibit similar phenotypes and that MUS-10 physically interacts with FZO1, though it is not clear if this interaction is direct or indirect. MUS-10 is an F-box protein that is thought to promote degradation of its substrate(s) through ubiquitination. For these reasons, we theorized that FZO1 is a substrate of MUS-10 (12). The ubiquitination of mitofusins or their homologues appears to be a conserved phenomenon from yeast to mammals. In budding yeast, the F-box protein Mdm30 ubiquititates the Fzo1 protein (63, 64). To facilitate mitochondrial outer membrane fusion, Fzo1 forms homodimers that tether two mitochondria. After GTP hydrolysis, Fzo1 is ubiquitinated by Mdm30 and then degraded following completion of outer membrane fusion (65, 66). Similar to the *mus-10* mutant of *N. crassa*, the *mdm30* mutant also harbors fragmented mitochondria (64, 67). Interestingly, high-throughput analysis revealed that the *mdm30* mutant is sensitive to UV (68). For *Drosophila* and mammals, it has been reported that MFN1 and MFN2 are ubiquitinated by parkin, an E3 ubiquitin ligase implicated in Parkinson’s disease, during mitophagy (69–71). It is thought that parkin may prevent Parkinson’s disease in humans by promoting mitophagy of damaged or depolarized mitochondria, which are believed to be deficient in mitochondrial fusion. Since homologues of Mdm30 and parkin are not found in *N. crassa*, it is plausible that MUS-10 may be a functional analogue of these proteins. If FZO1 is
ubiquitinated by MUS-10, and thus targeted for degradation by the proteasome, the FZO1 protein would be expected to accumulate in the mus-10 strain.

**Mutagen sensitivity.** There are no reports showing mutagen sensitivities of mitofusin mutants of yeast or higher organisms. For budding yeast, it has been revealed by a large-scale screening that the ugo1 mutant is sensitive to UV and MMS (72), and the mgm1 mutant is UV sensitive (68). Both the Ugo1 and Mgm1 proteins are mitochondrial fusion factors, with the former being fungus specific and the latter being homologous to mammalian OPA1. The link between mitochondrial fusion and mutagen hypersensitivity remains a mystery. Regulation of mitochondrial morphology influences various cellular processes, including ATP production, lipid metabolism, calcium signaling, apoptosis, and mitophagy (73). Recently, it was reported that mitophagy is important for mitochondrial quality control. In budding yeast, mitophagy signals induce phosphorylation of the mitophagy-specific protein Atg32, which then interacts with Atg1. Atg32 and Atg11 mutants are prone to mtDNA instability caused by oxidative stress (74). Thus, apoptosis and mitophagy may be closely related to mutagen sensitivity and senescence. However, it is interesting that the urs-5 and mus-10 mutants, which are defective in mitochondrial fusion, are hypersensitive to UV and MMS but not to oxidizing agents such as hydrogen peroxide and paraquat or to ionizing radiation, which causes severe DNA damage. Normally, N. crassa mutants deficient in DNA repair, such as homologous recombination or checkpoint mutants, are sensitive to UV, MMS, and ionizing radiation. Further analyses are required to resolve the missing link between mutagen sensitivity and mitochondrial morphology. Elucidation of these mechanisms should shed light on human mitochondrial diseases and senescence.

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