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The transcription factor ATF5 mediates a mammalian mitochondrial UPR

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SUMMARY

Mitochondrial dysfunction is pervasive in human pathologies such as neurodegeneration, diabetes, cancer and pathogen infections as well as during normal aging. Cells sense and respond to mitochondrial dysfunction by activating a protective transcriptional program known as the mitochondrial unfolded protein response (UPR^{mt}), which includes genes that promote mitochondrial protein homeostasis and the recovery of defective organelles [1, 2]. Work in *C. elegans* has shown that the UPR^{mt} is regulated by the transcription factor ATFS-1, which is regulated by organelle partitioning. Normally, ATFS-1 accumulates within mitochondria, but during respiratory chain dysfunction, high levels of ROS or mitochondrial protein folding stress, a percentage of ATFS-1 accumulates in the cytosol and traffics to the nucleus where it activates the UPR^{mt} [2]. While similar transcriptional responses have been described in mammals [3, 4], how the UPR^{mt} is regulated remains unclear. Here, we describe a mammalian transcription factor, ATF5, which is regulated similarly to ATFS-1 and induces a similar transcriptional response. ATF5 expression can rescue UPR^{mt} signaling in *atfs-1*-deficient worms requiring the same UPR^{mt} promoter element identified in *C. elegans*. Furthermore, mammalian cells require ATF5 to maintain mitochondrial activity during mitochondrial stress and to promote organelle recovery. Combined, these data suggest that regulation of the UPR^{mt} is conserved from worms to mammals.

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Author Contributions

CJF, AMS and CMH designed the experiments. CJF, AMS, MP, YFL, and NR performed the experiments. CJF, AMS and CMH interpreted the results and CJF and CMH wrote the manuscript. CJF performed the *C. elegans*, imaging and fractionation experiments and AS performed the mRNA quantification, cell proliferation and oxygen consumption experiments.

RESULTS AND DISCUSSION

Mitochondrial dysfunction is associated with normal aging and contributes to a number of pathologic conditions including Parkinson's, cancer, and infection. A number of stress responses and adaptations to mitochondrial dysfunction have been defined in mammalian systems such as the UPR^{mt}, which includes the induction of mitochondrial chaperones and proteases [4, 5]. A genetic screen in *C. elegans* identified the transcription factor ATFS-1 as a key regulator of the UPR^{mt}, which is regulated by organelle partitioning. ATFS-1 is normally imported into mitochondria [2, 6]. However, during mitochondrial dysfunction a percentage of ATFS-1 accumulates in the cytosol and subsequently traffics to the nucleus where it induces transcription of mitochondrial chaperones and proteases by binding the UPR^{mt} promoter element (UPR^{mt}E) [1, 2] as well as anti-bacterial innate immune genes [7]. Consistent with mediating a protective transcriptional response, worms lacking ATFS-1 incur respiratory defects during mitochondrial stress [1] and susceptibility to the pathogen *Pseudomonas aeruginosa* that perturbs mitochondrial function [7].

Considerable evidence suggests the UPR^{mt} is conserved in mammals where it was originally discovered. Expression of the mitochondrial protein ornithine transcarbamylase (OTC) lacking 84 amino acids rendering it unable to fold (ΔOTC), or exposure to ethidium bromide (EtBr) which depletes mitochondrial DNA (mtDNA), induces transcription of mitochondrial chaperone and protease genes in cultured cells [4, 8]. Furthermore, perturbation of mitochondrial ribosomes activates a similar transcription response in cultured cells and mice [3, 9], strongly suggesting the existence of a homologous regulatory mechanism to that described in *C.elegans*. Perhaps most intriguing, an element in the promoters of those genes induced in a mouse model of mitochondrial myopathy is nearly identical to the UPR^{mt}E to which ATFS-1 binds to induce chaperone and protease transcription in *C. elegans* [1, 10].

To identify potential regulators of a mammalian UPR^{mt}, we searched for bZip proteins homologous to ATFS-1 with potential mitochondrial targeting sequences (MTS). ATF4 and ATF5 had considerable homology within the bZip domain to ATFS-1, however ATF5 also had a putative, but relatively weak, MTS as determined by Mitoprot [11] (Figures 1A and S1A). Interestingly, several studies suggest a role for ATF5 during mitochondrial dysfunction. A recent transcription profiling study from patients with autosomal dominant ataxia caused by a mutation in a gene encoding a mitochondrial protease had increased *ATF5* transcripts [12], consistent with *atfs-1* transcripts being induced in *C. elegans* when the orthologous mitochondrial AAA protease (*spg-7*) is impaired [2]. Furthermore, mouse models of respiratory chain dysfunction caused by impaired mtDNA replication or a defective mitochondrial aspartyl-tRNA synthetase also caused induction of *ATF5* transcripts [10, 13].

To determine if either ATF4 or ATF5 can regulate a UPR^{mt}, the mammalian transcription factors were expressed in worms lacking ATFS-1. Identification of components that regulate the UPR^{mt} has been facilitated by the use of mitochondrial chaperone transcriptional reporter *C. elegans* strains [14] (Figure 1B). Induction of *hsp-60$_{pr}$::gfp* during mitochondrial stress requires both *atfs-1* and the UPR^{mt}E [1]. Interestingly, *atfs-1*-deletion worms expressing transgenic ATF5, but not worms expressing transgenic ATF4, were able to induce

the *hsp-60$_{pr}$::gfp* reporter during mitochondrial stress caused by depletion of a mitochondrial protein import component, *timm-23,* or protease, *spg-7* (Figure 1C). And, UPR^{mt} activation by ATF5 required the UPR^{mt}E in the *hsp-60* promoter (Figures 1B and D). ATF5 activation was mitochondrial stress-specific as perturbing endoplasmic reticulum (ER) protein folding with *ero-1*(RNAi) failed to activate *hsp-60$_{pr}$::gfp* in transgenic ATFS-1 or ATF5 worms (Figure 1C), but did activate the ER chaperone reporter *hsp-4$_{pr}$::gfp* (Figure S1B). Furthermore, transgenic ATF5 was unable to rescue induction of the ER UPR in worms lacking the ER stress-specific transcription factor XBP-1, further suggesting that ATF5 functions specifically during mitochondrial stress (Figure 1E).

Analysis of all mammalian gene promoters [15] revealed putative UPR^{mt}Es (Figure 1B) in the promoters of *HSP60* (*HSPD1*), *HSP10* (*HSPE1*), *mtHSP70* (*HSPA9*), the mitochondrial protease *LONP1*, as well as *ATF5* (data not shown) suggesting they may be regulated by ATF5. Exposure of HEK 293T cells to paraquat, which perturbs the respiratory chain causing toxic reactive oxygen (ROS) generation [16], resulted in increased transcription of *HSP60*, *mtHSP70*, *LONP1* and *HD-5*, a secreted anti-microbial peptide (Figure 2A), consistent with activation of a UPR^{mt}. Impressively, transcription of all four genes was significantly reduced when ATF5 was impaired by two different shRNAs (Figures 2A, S2A and S2B). Similarly, expression of ΔOTC, but not OTC (Figures S2C–G) caused modest induction of *mtHSP70, HSP10, and LONP1*, but not *HSP60* (Figure 2C) consistent with ΔOTC being less toxic than paraquat. However, all four transcripts were reduced in ΔOTC expressing cells when treated with either *ATF5* shRNA (Figures 2C and S2C–D), similar to *C. elegans* (Figure 2B). Lastly, *ATF5* transcripts were also increased during mitochondrial stress (Figure 2D) consistent with what has been observed for *atfs-1* in worms [1], for *Atf5* in mouse models of mitochondrial disease [10, 13] and *ATF5* in patient samples [12]. Of note, mRNAs encoding ER resident chaperones were not induced during mitochondrial stress in an ATF5-dependent manner (Figures S2H–I) suggesting ATF5 specifically promotes mitochondrial protein homeostasis.

We next sought to determine the impact of OXPHOS activity and mitochondrial membrane potential ($\Delta\psi$) on ATF5-dependent UPR^{mt} activation. Interestingly, simultaneous inhibition of respiratory chain complexes I, III and V, which dissipates $\Delta\psi$ [17] induced *LONP1* expression in an ATF5-dependent manner (Figure 2E) indicating that the UPR^{mt} can be activated upon loss of membrane potential. However, because separate treatment with either piericidin (complex I), antimycin (complex III), or oligomycin (complex V), which does not deplete $\Delta\psi$, also increased *LONP1* expression (Figure 2E), depletion of $\Delta\psi$ is not required for UPR^{mt} activation. Of note, unlike *LONP1* mRNA (Figure 2E), we did not detect an increase in the steady state protein expression of LONP1 during oligomycin treatment (Figure SJ), consistent with multiple post-transcriptional events occurring during mitochondrial stress concomitant to the UPR^{mt} including impaired mitochondrial protein import and subsequent degradation of those newly synthesized proteins [18, 19], the attenuation of protein synthesis [20, 21] and mitophagy [22][23].

Because ATF5 regulates mitochondrial chaperone and protease transcription during mitochondrial stress similarly to ATFS-1, we hypothesized ATF5 may also be regulated via organelle partitioning. ATF5-dependent transcription has been shown to coincide with its

nuclear accumulation [24, 25], consistent with the presence of a nuclear localization sequence (Figure 1A). Therefore, we sought to determine if ATF5 localizes to mitochondria in the absence of mitochondrial stress. Subcellular fractionation in *C. elegans* indicated that in the absence of stress, ATF5 co-fractionated with a mitochondrial protein (Figure 3A), consistent with ATF5 being inactive in the absence of stress (Figure 1C).

Endogenous ATF5 is difficult to detect in cultured mammalian cells as translation of the *ATF5* transcript is impaired by the presence of upstream open reading frames (uORFs) [26], and its relatively short half-life [27]. To increase ATF5 expression, HeLa cells were cultured in the presence of a proteasome inhibitor that leads to phosphorylation of the translation initiation factor eIF2α and increases synthesis of proteins encoded by uORF containing mRNAs [28]. Bortezomib treatment resulted in increased expression of ATF5, which was impaired by *ATF5* shRNA (Figure 3B). Interestingly, ATF5 was enriched in the mitochondrial fraction further supporting localization of ATF5 in mitochondria in the absence of mitochondrial stress. Of note, we were unable to detect cleavage of the MTS upon import into mitochondria (Figure 3B), suggesting the MTS remains intact not unlike several other mitochondrial proteins [29].

Because we were only able to detect endogenous ATF5 in cultured cells upon proteasome inhibition (Figure 3B), we expressed ATF5::GFP via the CMV promoter in HeLa cells. Given the strong over-expression, considerable ATF5::GFP localized to the nucleus as expected, but ATF5::GFP also co-localized with mitochondria (Figures 3C and D), unlike GFP fusion proteins directed to the cytosol or nucleus (Figures 3C and D). Unlike in cultured cells, ATF5 is highly expressed in mouse and human liver cells [30], which coincides with an isoform of *ATF5* mRNA lacking uORFs [31]. Interestingly, sucrose fractionation of mouse liver homogenates demonstrated co-fractionation of ATF5 with a mitochondrial, but not an ER-resident protein (Figure 3E). Given the difficulties in detecting ATF5 perhaps due to its low expression and short half-life [26, 32], it has been challenging to observe stress dependent shifts in ATF5 localization, but because ATF5-dependent transcription is induced during mitochondrial stress and ATF5 localizes to mitochondria in the absence of stress, our data are consistent with ATF5 being regulated post-translationally similarly to ATFS-1.

Lastly, we sought to determine the role of ATF5 in protecting or maintaining mitochondrial function. Interestingly, in HEK 293T cells, knockdown of ATF5 reduced basal respiration, overall respiratory capacity and maximal respiration (Figures 4A–B, S3A–B) suggesting a basal role for ATF5 in mitochondrial maintenance in these cells. *ATF5* knockdown also impaired cell proliferation specifically in cells expressing ΔOTC (Figures 4C–D), further suggesting ATF5 promotes mitochondrial function during stress. We next examined the role of ATF5 during the recovery from mitochondrial stress caused by depletion of mitochondrial genomes (mtDNAs) via EtBr exposure. mtDNA was depleted to ~10% of normal levels in both control and *ATF5* shRNA cells (Figures 4E and 4F). Upon EtBr removal, both cell types recovered mtDNA levels at similar rates (Figure 4E), suggesting ATF5 does not affect mtDNA replication. However, the *ATF5* shRNA cells proliferated much more slowly (Figures 4F and S3C–D) suggesting ATF5 and the regulation of mitochondrial protein homeostasis machinery promotes the recovery from mitochondrial stress. Furthermore,

ATF5 shRNA reduced the steady state expression of *HSP60*, *mtHSP70*, *LONP1* and impaired cell growth (Figures 4G and S3E) in an oncocytic cell line harboring multiple mtDNA lesions that impair respiratory chain activity further supporting the role for ATF5-dependent transcription during mitochondrial dysfunction [33].

Combined, our data suggest that ATF5 regulates a UPR^{mt} in mammalian cells that is similar to the response regulated by ATFS-1 in *C. elegans*. Our data support a model that when expressed, ATF5 localizes to mitochondria in the absence of stress. However, during mitochondrial stress, there is induction of mitochondrial protective transcripts, or a UPR^{mt}, in an ATF5-dependent manner. This role is consistent with a previously established function of ATF5 as being anti-apoptotic by inducing *BCL-2* transcription [34] and promoting survival of a number of cancer cells including glioblastomas [35, 36], which are known to have mitochondrial dysfunction [37]. Of note, our work also suggests an interaction between the UPR^{mt} and the integrated stress response (ISR) because increased eIF2α-phosphorylation results in preferential ATF5 synthesis [26]. This is consistent with several studies demonstrating increased eIF2α phosphorylation during mitochondrial dysfunction mediated by the kinases GCN2 and PERK [38, 39] and suggests an additional layer of regulation in addition to organelle partitioning. Our findings indicate a protective role for ATF5 during mitochondrial dysfunction by regulating a UPR^{mt}.

Experimental Procedures

A full description of the experimental procedures can be found in the Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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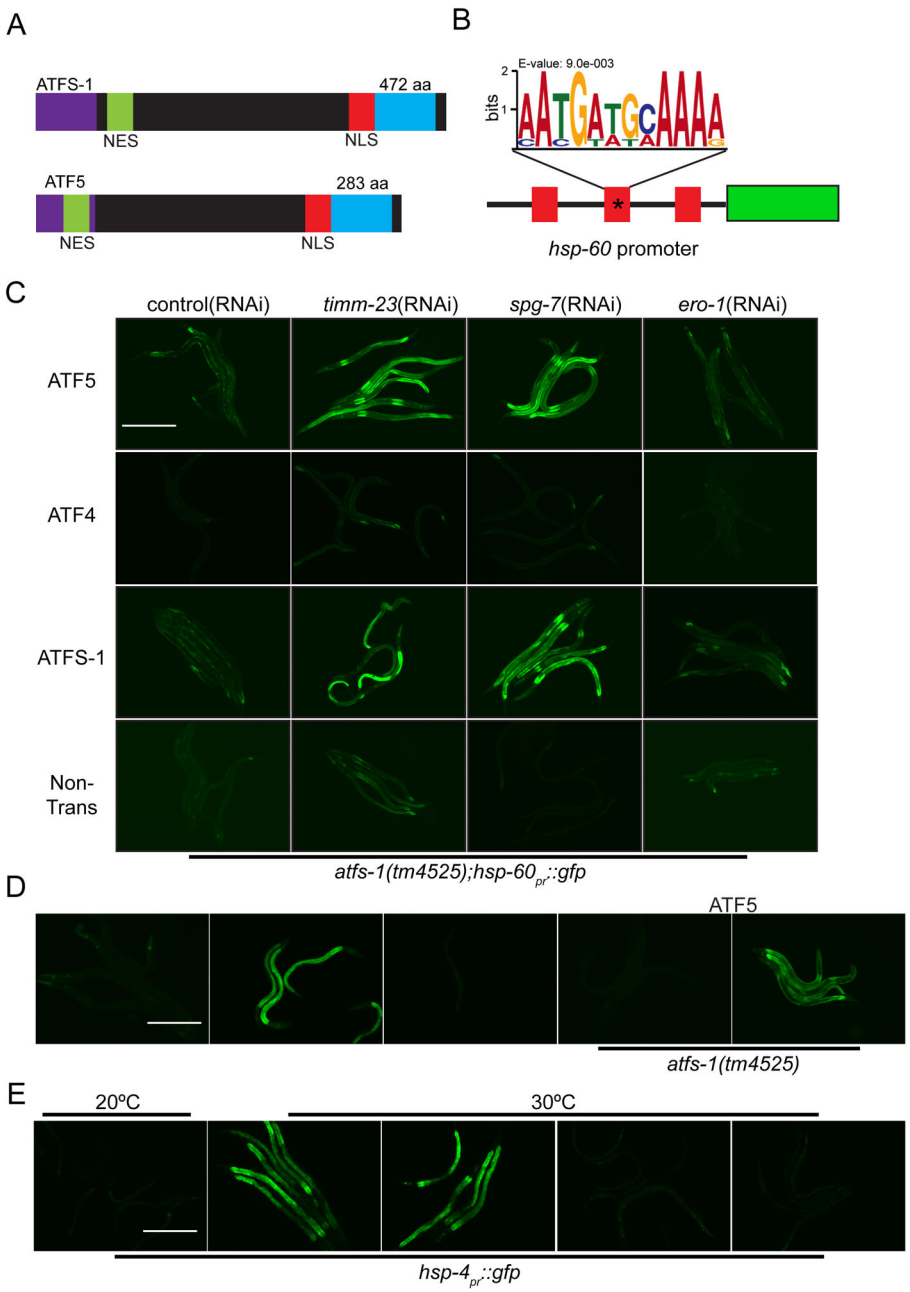

Figure 1. Expression of ATF5 rescues UPR^{mt} activation in worms lacking ATFS-1

(A) Schematic comparing the bZip transcription factors ATFS-1 and ATF5 including the mitochondrial targeting sequence (MTS), nuclear export sequence (NES) and the nuclear localization sequence (NLS).

(B) Schematic of the *hsp-60$_{pr}$::gfp* reporter highlighting the three UPR^{mt} elements in the promoter. The mutated element used in Figure 1D is marked with an asterisk (*).

(C) Photomicrographs of *atfs-1(tm4525);hsp60$_{pr}$::gfp* worms expressing transgenic ATF5, ATF4 or ATFS-1 and raised on control, *timm-23*, *spg-7*, or *ero-1*(RNAi). Scale bar, 0.5 mm.

(D) Photomicrographs of wildtype and *atfs-1(tm4525)* worms expressing either *hsp-60*$_{pr}$*::gfp* or *hsp-60*$_{pr}$*::gfp* lacking a UPR^{mt}E (*) (Figure 1B) raised on control or *spg-7*(RNAi). Worms in the right two panels express transgenic ATF5. Scale bar, 0.5 mm. (E) Photomicrographs of control or transgenic ATF5 expressing *hsp-4*$_{pr}$*::gfp* worms, raised on control or *xbp-1*(RNAi) incubated at 20°C or 30°C. Scale bar, 0.5 mm. See also Figure S1.

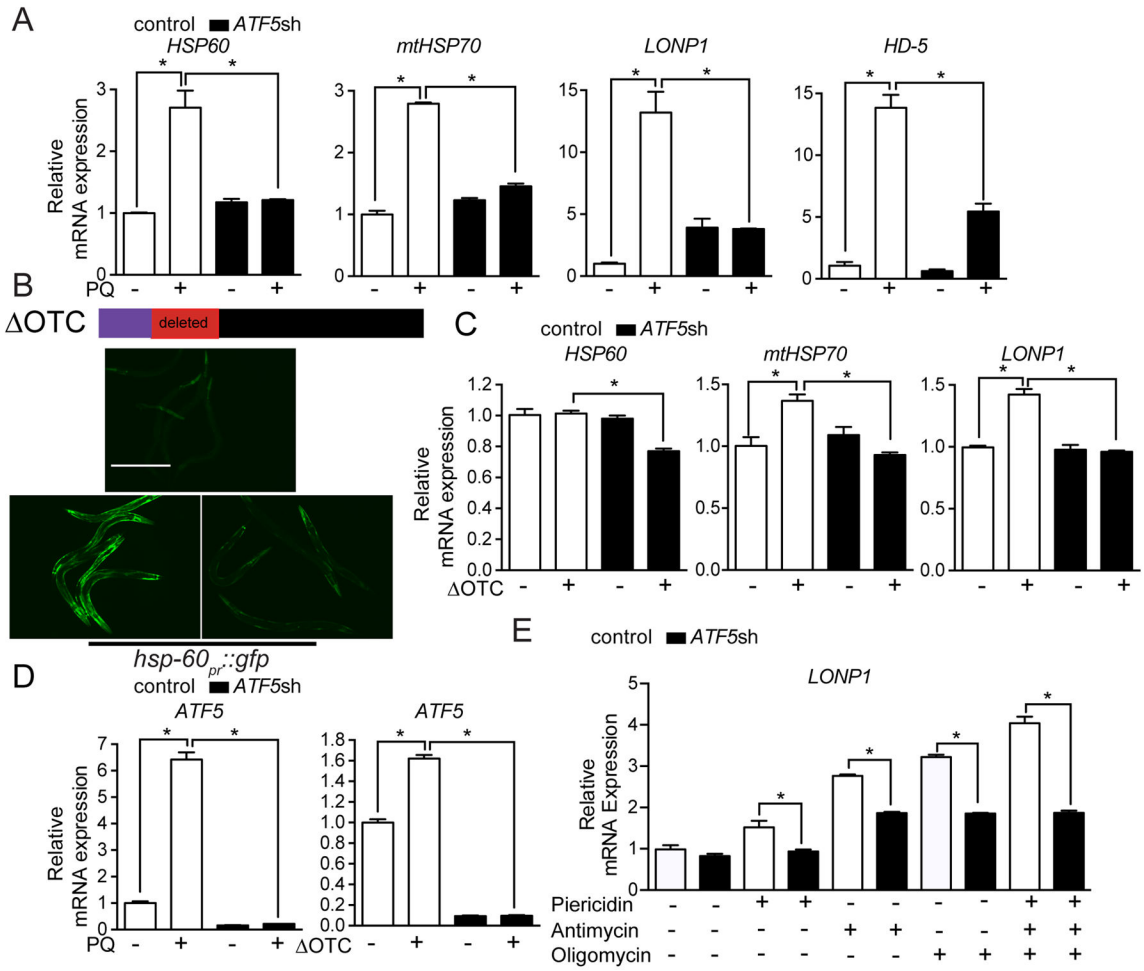

Figure 2. ATF5 is required for UPR^{mt} activation in mammalian cells

(A) Expression levels of *HSP60*, *mtHSP70*, *LONP1*, and *HD-5* mRNA in control or *ATF5* shRNA HEK 293T cells with or without paraquat (PQ) (n=3, mean ± SEM, *p<0.05).

(B) Schematic showing ΔOTC construct. Photomicrographs of *hsp-60$_{pr}$::gfp* worms raised on control or *atfs-1*(RNAi) with or without ΔOTC expression via the muscle-specific *myo-3* promoter. Scale bar, 0.5 mm.

(C) Expression levels of *HSP60*, *mtHSP70*, and *LONP1* mRNA in control or *ATF5* shRNA HEK 293T cells with or without ΔOTC expression (n=3, mean ± SEM, *p<0.05)

(D) Expression levels of *ATF5* mRNA in control or *ATF5* shRNA HEK 293T cells with or without paraquat (PQ), or expressing ΔOTC (n=3, mean ± SEM, *p<0.05).

(E) Expression levels of *LONP1* mRNA in control or *ATF5* shRNA HEK 293T cells treated with oligomycin, antimycin, piericidin, or all three inhibitors (n=3, mean ± SEM, *p<0.05).

See also Figure S2.

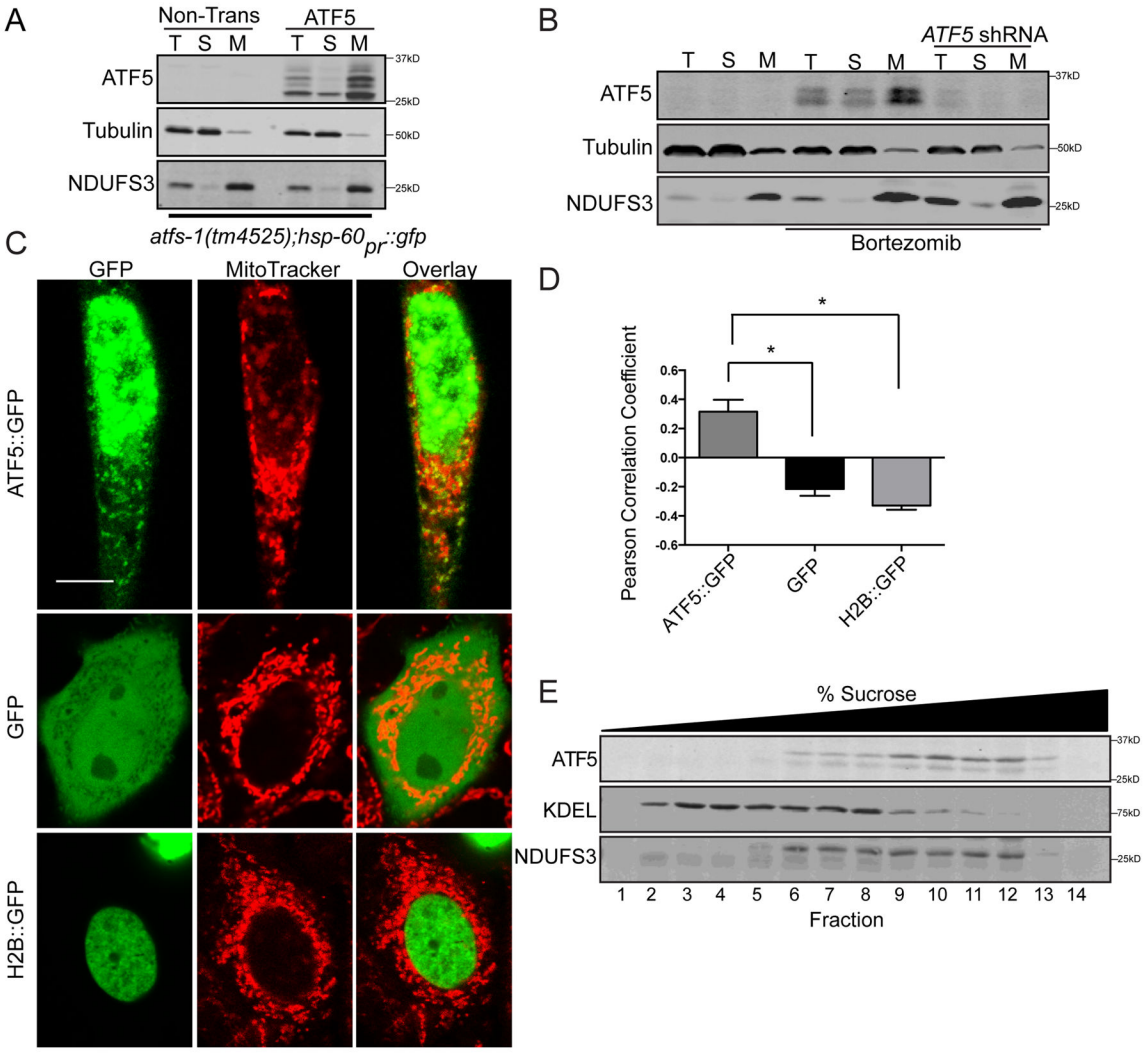

Figure 3. ATF5 localizes to mitochondria and nuclei

(A) Immunoblots of lysates from control or ATF5 expressing *atfs-1(tm4525);hsp60$_{pr}$::gfp* worms following fractionation into total lysate (T), postmitochondrial supernatant (S), and mitochondrial pellet (M). NDUFS3 serves as a mitochondrial marker and tubulin as a cytosolic marker.

(B) Immunoblots of control or *ATF5* shRNA HeLa cells treated with DMSO or Bortezomib and fractionated into total lysate (T), postmitochondrial supernatant (S), and mitochondrial pellet (M).

(C) Photomicrographs of HeLa cells expressing either ATF5::GFP, Histone 2B::GFP (H2B::GFP), or GFP and stained with Mitotracker. Scale bar, 0.01 mm.

(D) Pearson Correlation Coefficient [40] of co-localization of ATF5::GFP, Histone 2B::GFP (H2B::GFP), or GFP and MitoTracker (Figure 3C) (n=5, mean ± SEM, *p<0.05).

(E) Immunoblot of mouse liver fractions following centrifugation on a sucrose gradient. Endogenous KDEL serves as an ER marker and NDUFS3 as a mitochondria marker.

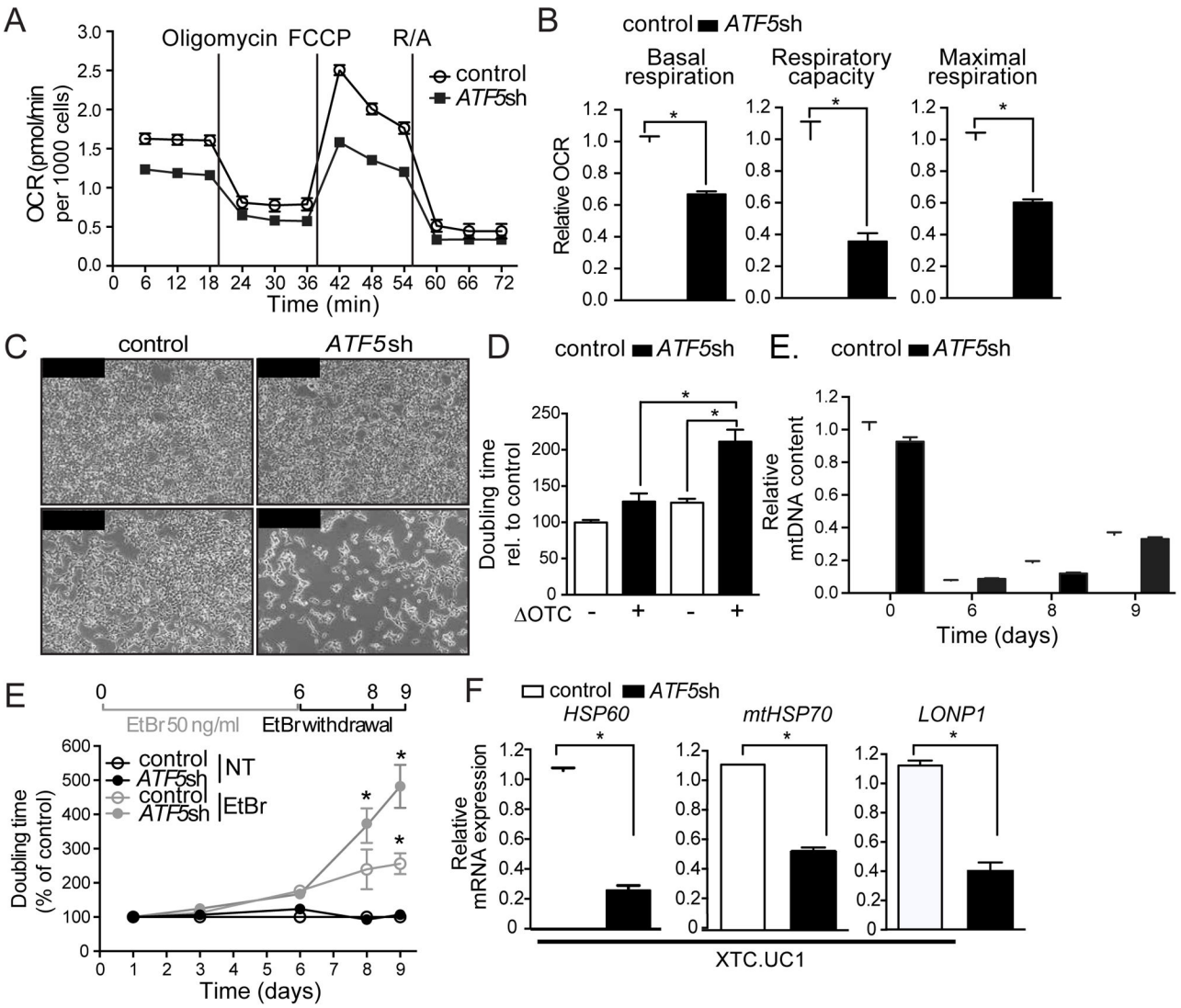

Figure 4. ATF5 promotes proliferation and recovery from mitochondrial stress

(A–B) Oxygen consumption rates (OCR) in control or *ATF5* shRNA HEK 293T cells (n=15, mean ± SEM,*p<0.05).

(C–D) Photomicrographs (C) and doubling times (D) of control or *ATF5* shRNA HEK 293T cells, with or without ΔOTC expression. Scale bar, 0.1 mm (n=3-7, mean ± SEM *p<0.05).

(E) mtDNA quantification of control and *ATF5* shRNA HEK 293T after 6 days of EtBr treatment, 4 days of withdrawal (n=3, mean ± SEM).

(F) Time course of doubling times of control and *ATF5* shRNA HEK 293T cells following 6 days of mtDNA depletion by EtBr treatment (n=3, mean ± SEM, *p<0.05).

(G) Expression levels of *HSP60*, *mtHSP70*, or *LONP1* mRNA in control or *ATF5* shRNA XTC.UC1 cells (n=3, mean ± SEM, *p<0.05). See also Figure S3.