Mitochondrial Lon protease regulates mitochondrial DNA copy number and transcription by selective degradation of mitochondrial transcription factor A (TFAM)

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Lon is the major protease in the mitochondrial matrix in eukaryotes, and is well conserved among species. Although a role for Lon in mitochondrial biogenesis has been proposed, the mechanistic basis is unclear. Here, we demonstrate a role for Lon in mtDNA metabolism. An RNA interference (RNAi) construct was designed that reduces Lon to less than 10% of its normal level in Drosophila Schneider cells. RNAi knockdown of Lon results in increased abundance of mitochondrial transcription factor A (TFAM) and mtDNA copy number. In a corollary manner, overexpression of Lon reduces TFAM levels and mtDNA copy number. Notably, induction of mtDNA depletion in Lon knockdown cells does not result in degradation of TFAM, thereby causing a dramatic increase in the TFAM:mtDNA ratio. The increased TFAM:mtDNA ratio in turn causes inhibition of mitochondrial transcription. We conclude that Lon regulates mitochondrial transcription by stabilizing the mitochondrial TFAM:mtDNA ratio via selective degradation of TFAM.

Results

Overexpression of Lon Reduces TFAM Levels and mtDNA Copy Number. Mitochondrial localization of the Drosophila Lon gene product (CG8798) was confirmed in Schneider cells by fluorescence microscopy (Fig. S1). Next, Drosophila Lon was subcloned into the inducible expression vector pMt/Hy under the control of the metallothionein promoter. The resulting expression vector, pMt/Lon/Hy, was introduced into Schneider cells, and stable cell lines harboring this plasmid were cultured in media with or without 0.2 mM CuSO4. After 10 d of incubation in the presence of copper, immunoblot analysis indicated a fivefold increase in Lon relative to that in the uninduced control cells (Fig. L1). In contrast, expression of β-tubulin, used as a control protein, was unchanged. Levels of protein components of the mitochondrial nucleoid were measured by immunoblotting of cells carrying no plasmid, pMt/Hy, or pMt/Lon/Hy.

Overexpression of Lon reduced the level of TFAM to 75% of that in the control cells (Fig. 1 A, B). Levels of other proteins localized in mitochondrial nucleoids, including mtTFB2, mtDNA helicase, pol γ-α, and mtSSB, were not changed. We used Southern blots to quantify relative mtDNA copy number in the Lon overexpression cells. We found that the relative mtDNA copy number in the overexpression cells was ~0.7-fold of that in the control cells (Fig. 1C). Northern blots were used to quantify the relative expression of the Cytb, ND4, and 12S rRNA genes in cells grown for 10 d in the presence or absence of copper. Overexpression of Lon did not show any significant changes on these mitochondrial transcript levels as compared to the control cells (Fig. 1D). Furthermore, TFAM mRNA levels were unchanged by Lon overexpression, indicating that the reduction in TFAM protein levels in the knockdown cells does not result from the reduction of TFAM mRNA.

RNAi-Dependent Knockdown of Lon Increases TFAM, Mitochondrial DNA Copy Number, and Mitochondrial Transcription. We reduced the abundance of Lon by expressing a metallothionein-inducible Lon-targeted RNAi species from the plasmid pMt/invLon/Hy. The RNA species produced a form of dsRNA hairpin homolo-

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Fig. 1. Expression of Drosophila Lon protease in Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Hy (vector) or pMt/Lon/Hy (Lon WT) were cultured for 10 d in the presence or absence of 0.2 mM CuSO4. (A) Protein extracts (20 μg) were fractionated by 7.5%, 10.5%, or 13.5% SDS-PAGE, transferred to nitrocellulose filters and probed with antibodies against Lon protease, TFAM, mtTFB2, mtDNA helicase, pol γ-α, mtSSB, ATPase β, or β-tubulin as indicated. (B) The TFAM/β-tubulin ratio was quantitated by normalizing TFAM protein levels to β-tubulin protein levels as described under Materials and Methods. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates P < 0.05 in comparison to control. (C) Total DNA (10 μg) was extracted from Schneider cells or Schneider cells carrying pMt/Hy or pMt/Lon/Hy that were cultured for 10 d in the presence of 0.2 mM CuSO4. DNA was digested with XhoI, fractionated in a 0.7% agarose/TBE gel, and then blotted to a nylon membrane. The membrane was hybridized with a radiolabeled probe for Cytb, and then stripped and rehybridized with radiolabeled probe for the histone gene cluster as a control. The relative mtDNA copy number was quantitated as described under Materials and Methods. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates P < 0.05 in comparison to control. (D) Total RNA (10 μg) was extracted from Schneider cells or Schneider cells carrying pMt/Hy or pMt/Lon/Hy after 10 d of culture in the presence or absence of 0.2 mM CuSO4. RNA was fractionated in a 1.2% agarose/formaldehyde gel, and then blotted to a nylon membrane. The membrane was hybridized with a radiolabeled probe for the histone gene cluster as a control. The nuclear transcript was quantitated as described in the legend to Fig. 1. (A) Immunoblot analysis was carried out as described in the figure legend to Fig. 1A. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates P < 0.05 in comparison to control.

Fig. 2. Expression of Drosophila Lon-targeted RNAi in Schneider cells. Schneider cells with no plasmid (control) or carrying pMt/Hy (vector) or pMt/invLon/Hy (Lon RNAi) were cultured for 10 d in the presence or absence of 0.2 mM CuSO4. (A) Immunoblot analysis was carried out as described in the figure legend to Fig. 1A. (B) The TFAM/β-tubulin ratio was quantitated by normalizing TFAM protein levels to β-tubulin protein levels as described under Materials and Methods. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates P < 0.05 in comparison to control. (C) Relative mtDNA copy number was determined as described in the figure legend to Fig. 1C. Error bars indicate means ± standard error of three independent experiments. The asterisk indicates P < 0.05 in comparison to control. (D) Northern blot analysis using 12S rRNA, ND4, Cytb, RP49, and TFAM was carried out as described in the legend to Fig. 1D.
tein in the knockdown cells does not result from an increase in the steady-state level of TFAM transcripts.

Lon Regulates mtDNA-Dependent TFAM Degradation in Schneider Cells. Our data show that Lon regulates TFAM levels and mtDNA copy number. Because of the interdependent relationship of TFAM and mtDNA, we asked whether or not Lon degrades TFAM directly. To do so, we cultured the cell lines in the presence of ethidium bromide (EtBr), an inhibitor of mtDNA replication. In the control cells, EtBr treatment results in a rapid reduction of mtDNA copy number, and TFAM protein levels were also reduced, albeit more slowly than that of mtDNA (Fig. 3A, B). At the same time, TFAM mRNA levels were unchanged (Fig. S3). Because the levels of other mitochondrial nucleoid proteins were not affected by mtDNA depletion with EtBr, we conclude that TFAM is depleted selectively following mtDNA reduction. In the Lon overexpression cells, EtBr treatment also resulted in a reduction of mtDNA copy number, and TFAM protein levels were also reduced (Fig. 3A). EtBr treatment also caused mtDNA depletion in the Lon knockdown cells. However, in this case, the relative level of TFAM protein was increased 1.2-fold (Fig. 3A), while again TFAM mRNA levels were unchanged (Fig. S3). Similar to the control cells, the levels of other mitochondrial nucleoid proteins were unchanged in the EtBr treated Lon knockdown cells.

To demonstrate conclusively the proteolytic role of Lon in mtDNA-dependent TFAM degradation, we established a cell line expressing a Lon mutant carrying a S880A amino acid substitution, in which the conserved serine in the proteolytic active site was replaced by alanine. The cell line expressing Lon S880A showed severe retardation of TFAM degradation following mtDNA depletion, likely because overexpression of Lon S880A results in a dominant negative phenotype that is caused by the formation of mixed oligomeric forms (Fig. S4). Taken together, our data show clearly that Lon is responsible for specific degradation of TFAM.

What Is the Physiological Role of Lon Degradation of TFAM? We sought to investigate the functional significance of the specific degradation of TFAM in mtDNA-depleted cells. In control cells treated with EtBr, the TFAM:mtDNA ratio was raised transiently, and then reverted to normal levels within 6 d (Fig. 3C). However, Lon knockdown cells did not recover a normal TFAM:mtDNA ratio. In mammalian cells, overexpression of TFAM causes suppression of mitochondrial transcription (36). We confirmed this phenomenon in Schneider cells overexpressing TFAM under the control of the metallothionein promoter (Fig. S5), and find that suppression occurs in cells containing a TFAM:mtDNA ratio >2 (Fig. 4). Interestingly, the highest overexpression level showed depletion of mtDNA copy number in addition to transcriptional suppression. We thus hypothesized that Lon regulates mtDNA transcription by stabilizing the cellular TFAM:mtDNA ratio. To document this hypothesis, we measured mitochondrial transcript levels in mtDNA-depleted cells. Because EtBr also inhibits mtDNA transcription, we instead induced mtDNA depletion by knockdown of mtDNA replication factors and in particular, the catalytic subunit of mitochondrial DNA polymerase and the mtDNA helicase. Control and Lon knockdown cells were cultured for 10 d with dsRNA targeted against these proteins or GFP as a control, and the relevant protein levels were then evaluated by immunoblotting (Fig. 5A). The cells cultured with GFP dsRNA showed no change in these protein levels, whereas in the cell lines cultured with the mtDNA helicase or mtDNA polymerase dsRNAs, mtDNA copy number was reduced to ~60% of that in the control cells (Fig. 5B). After the dsRNA treatments, the TFAM levels decreased to 75% in control cells, but increased 1.2-fold in the Lon knockdown cells (Fig. 5A). After the dsRNA treatments, the TFAM:mtDNA ratio in the control cells was increased ~1.3-fold relative to the control cells alone, and the ratio in the Lon RNAi cells was increased ~2.5-fold (Fig. 5C). Mitochondrial transcripts in the control cells were unchanged with or without dsRNA treatment (Fig. 5D). However, the transcript levels in mtDNA-depleted Lon RNAi cells were reduced to 47%–60% of those in the mtDNA-depleted cells that showed transcript levels equivalent to 66%–84% of the
Control experiments. The asterisk indicates differences to that of RP49. Error bars indicate means ± standard error of two independent experiments. Theamber indicates that TFAM/mtDNA ratio.

**Discussion**

We have established that Lon knockdown cell lines express <10% of endogenous Lon protein levels, yet these cells grow for at least 6 mo. In human fibroblast cells, depletion of Lon over 4 d resulted in apoptotic cell death (37, 38), whereas Lon knockdown in human colon carcinoma cells allows survival for at least 15 d (18). Thus, the effects of Lon depletion may be species- or cell type-specific. As in a recent report with human rhabdomyosarcoma cells (39), depletion of Lon protease in *Drosophila* Schnei
der cells results in an increase in the levels of oxidized proteins in mitochondria, indicating that Lon is responsible for degradation of oxidized mitochondrial proteins, and suggesting that variations in cell viability as a result of Lon depletion may reflect varying cellular tolerances for oxidative damage to mitochondrial proteins.

It is known that TFAM protein levels are reduced coincident with mtDNA depletion in animal cells, but the mechanism for this phenomenon is unclear (31). Here, we show that Lon is responsible for degradation of TFAM upon mtDNA depletion. Moreover, Lon may be also responsible for TFAM degradation under normal conditions, because the cellular level of TFAM varies in concert with Lon levels. These findings imply that TFAM turnover is strongly dependent on Lon protease function. In addition to TFAM, we found that the mtTFB2 level is increased in Lon knockdown cells, so it may also be a specific substrate for the Lon protease. Other mitochondrial biogenesis proteins tested were unchanged appreciably in either Lon knockdown or overexpression cells. In *Escherichia coli*, some substrates for Lon overlap those of other AAA+ proteases such as the ClpP protease; notably, the ClpP ortholog in animal cells comprises another major protease in the mitochondrial matrix space (2, 3, 40). Therefore, the depletion of mitochondrial Lon may be compensated partially by mitochondrial ClpP protease. Alternatively, the residual Lon in knockdown cells may be sufficient to degrade its protein targets.

We found that mitochondrial transcripts in Lon knockdown cells are increased moderately in association with an increase of TFAM and mtTFB2 levels. Previous studies showed that the relative levels of mtDNA and TFAM are not critical to observe stimulation of mitochondrial transcription in *Drosophila* Kc167, and Schneider cells (29, 35). We suggest that in contrast, increase of mtTFB2, which is essential for mitochondrial transcription, may be responsible for up-regulation of mitochondrial transcription.

We show that degradation of TFAM by Lon protease is facilitated by mtDNA depletion. Interestingly, a similar phenomenon was reported in *Bacillus subtilis*. *B. subtilis* LonA, which is the ortholog of Lon, is involved in degradation of the structural maintenance of chromosomes protein (SMC), and the degradation of SMC is facilitated by DNAase treatment (41). Although the mechanisms by which Lon recognizes its target proteins are not well understood, a recent report showed that *E. coli* Lon recognizes specific aromatic residue-rich sequences that are hidden in the hydrophobic cores of native structures, but are accessible in unfolded structures (42). Interestingly, the HMG boxes in TFAM contain four conserved aromatic residues within a hydrophobic core, and these residues may be masked when TFAM binds to mtDNA (25, 27, 29). Another possible explanation is that TFAM not bound to mtDNA becomes exposed to oxidative stress, whereas TFAM bound to mtDNA comprises part of the core of the mitochondrial nucleoids, and is thus surrounded by other proteins (43). Mitochondrial Lon has the ability to bind DNA and localizes in mitochondrial nucleoids (15–18). Our current hypothesis is that excess free TFAM is degraded by Lon before the TFAM binds DNA. Alternatively, it seems possible, albeit more complicated, that excessive DNA compaction resulting from binding of high TFAM levels signals the degradation of TFAM by DNA-bound Lon. Further experiments are warranted to address these and other possibilities, and to clarify the link between the DNA-binding activity of Lon and TFAM turnover.

Suzuki and colleagues showed that mtDNA copy number was unchanged after 15 d of Lon knockdown in human colon carcinoma cells (18). This model differs from ours, in which we observe an increase in mtDNA copy number. We established our Lon knockdown cells over a period of 8 wks and because of leaky expression from the inducible promoter in the RNAi vector, Lon depletion was already ongoing prior to the 10 d induction.
period. Thus, one possible explanation for the difference we observe is that TFAM accumulation may be slower in their model and consequently, increased mtDNA copy number might not have been apparent. Another possible explanation is that compensation by other proteases such as ClpP (2, 40) might effect TFAM degradation in the Lon-depleted human colon carcinoma cells.

We found that upon EtBr treatment, the TFAM:mtDNA ratio is nearly restored within 6 d in both control and Lon overexpressing cells, whereas restoration did not occur in Lon knockdown cells. Moreover, TFAM turnover resulting from mtDNA depletion by EtBr treatment is strongly reduced in cells overexpressing TFAM. With the example of TFAM, we provide direct evidence that TFAM turnover resulting from mtDNA depletion, whereas Lon knockdown cells showed a similar reduction in mitochondrial transcripts. Upon knockdown of mtDNA helicase or mtDNA polymerase, mitochondrial transcription levels are unchanged in control cells upon mtDNA depletion, whereas Lon knockdown cells showed a similar reduction in mitochondrial transcripts. Upon knockdown of mtDNA helicase or mtDNA polymerase, the TFAM:mtDNA ratio is ~1.3 in control cells and >2 in Lon knockdown cells, consistent with the observations in TFAM overexpression cells. Because there are environmental conditions under which mtDNA copy number is known to vary, such as during development and upon drug treatment (44, 45), transient Lon degradation of excess TFAM would be important to maintain normal mitochondrial transcription levels. We conclude that the TFAM:mtDNA ratio is crucial for both mtDNA biogenesis and homeostasis, and that Lon stabilizes the TFAM:mtDNA ratio by degradation of excess TFAM. With the example of TFAM, we provide direct evidence of the physiological role of Lon protease activity in mtDNA maintenance in animal cells, warranting future study of its potential involvement in the surveillance and turnover of other proteins involved in mtDNA replication, transcription, and translation.

Materials and Methods
Preparation of Lon Antibody. A recombinant protein corresponding to amino acids Asp613 to Ser836 of Drosophila Lon (CG8798) was used to immunize rabbits to obtain polyclonal antibody.

Preparation of Inducible Plasmodis Expressing Lon, TFAM, and Lon-Targeted RNAi. The plasmid pMt/Lon/Hy, in which Lon is regulated by the metallothio- nein promoter and the plasmid pMt/inv/Lon/Hy, which carries an inverted repeat of a nucleotide sequence from Lon cDNA that is transcribed from the metallothionein promoter were constructed as described in the SI Text. Plasmid pMt/TFAM/Hy is as described previously (35).

Generation and Induction of Stable Cell Lines. Drosophila Schneider S2 cells were cultured at 25°C in Drosophila Schneider Medium (Invitrogen) supplemented with 10% FBS. Cells were subcultured to 3 × 10^6 cells/ml every 5 days. Cells were transfected using Effectene (QIAGEN). Hygromycin-resistant cells were selected with 200 μg/ml hygromycin. Cells were passaged for 8 wks in hygromycin-containing medium and then cultured in standard medium. The cell lines were grown to a density of 3 × 10^6/ml and then treated with 0.2 mM CuSO_4 to induce expression from the metallothionein promoter.

Immunoblotting. Total cellular protein (20 μg per lane) was fractionated by 13.5%, 10.5%, or 7.5% SDS-PAGE and transferred to nitrocellulose filters. Immunoblotting was performed as described previously (46). Protein bands were visualized using ECL Western blotting reagents (Amersham). ECL luminescence was quantified on a Kodak Image Station 4000R. Antibodies against Drosophila mtSSB (47), TFAM (29), Pol γ–α (48), mtDNA helicase (32), ATPase β (32), mtTFB2 (35), and β tubulin (E7) (Developmental Studies Hyridoma Bank) were prepared and used as described. The ratio of the signals for TFAM and β tubulin was used to estimate the relative protein levels of TFAM. The TFAM immunoblotting experiments shown in Figs. 1, 2, 3, and 5 were performed two or three times with each of the two or three independent cell lines carrying each plasmid construct, including control (no plasmid). The data presented represent one such experiment, and the quantitation is provided for the duplicate or triplicate experiments from one of two or three cell lines.

Northern and Southern Blotting. Northern blotting was performed as described previously (46), and the data were analyzed using a Phospholmage (Molecular Dynamics). The signal for R49 was used to normalize mitochondrial transcripts.

Southern blotting was performed as described previously (32), and the data were analyzed using a Phospholmage (Molecular Dynamics). Blots were probed with radioactively labeled DNAs for the mitochondrial gene Cytb and the nuclear gene gene cluster. The ratio of the signals for these two genes was used to estimate the relative copy number of mtDNA. The Northern and Southern blot experiments shown in Figs. 1, 2, 3, 4, and 5 were performed two or three times with each of the two or three independent cell lines carrying each plasmid construct, including control (no plasmid). The data presented represent one such experiment, and the quantitation is provided for the duplicate or triplicate experiments from one of two or three cell lines.

RNA Interference. To generate double-stranded RNA (dsRNA) for RNAi, sequences directed against the protein to be silenced were amplified by PCR from each cDNA. Each primer used in the PCR contained a T7 RNA promoter followed by sequences specific for the targeted genes. The following primer sets were used for each protein: mtDNA helicase for GTAATACGACTCATA-TAGGGGCTAGAAATGAGAGCCG and GTAATACGACTCATAAGGGGAT- TGCAGTACAGAACGG; DNA polymerase γ for TAATACGACTCATAAGGG- GTGCTCAGTACAGGGCG and TAATACGACTCATAAGGGGAT-GGTCAATACAGAG; GFP for GTAATACGACTCATAAGGGGAAAGACAGATT- CCTACGG and GTAATACGACTCATAAGGGCGTCTAGTGGTGGCG. PCR products were used as templates for in vitro transcription using the T7 Mega- script RNAi kit (Ambion). 3 × 10^6 S2 Drosophila tissue culture cells were plated into a T25 flask in 5 mL of medium without FBS. 100 μg of dsRNA was added and mixed by swirling. After 30 min, 5 mL of media containing 20% FBS was added. The cells were collected after 5 d culture and repeated dsRNA treatment. After 5 d after dsRNA treatment, the cells were harvested for the analysis.

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