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Phosphorylation of Human TFAM in Mitochondria Impairs DNA Binding and Promotes Degradation by the AAA+ Lon Protease

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SUMMARY

Human mitochondrial transcription factor A (TFAM) is a high-mobility group (HMG) protein at the nexus of mitochondrial DNA (mtDNA) replication, transcription, and inheritance. Little is known about the mechanisms underlying its posttranslational regulation. Here, we demonstrate that TFAM is phosphorylated within its HMG box 1 (HMG1) by cAMP-dependent protein kinase in mitochondria. HMG1 phosphorylation impairs the ability of TFAM to bind DNA and to activate transcription. We show that only DNA-free TFAM is degraded by the Lon protease, which is inhibited by the anticancer drug bortezomib. In cells with normal mtDNA levels, HMG1-phosphorylated TFAM is degraded by Lon. However, in cells with severe mtDNA deficits, nonphosphorylated TFAM is also degraded, as it is DNA free. Depleting Lon in these cells increases levels of TFAM and upregulates mtDNA content, albeit transiently. Phosphorylation and proteolysis thus provide mechanisms for rapid fine-tuning of TFAM function and abundance in mitochondria, which are crucial for maintaining and expressing mtDNA.

INTRODUCTION

Human mitochondrial transcription factor A (TFAM) is essential for mitochondrial DNA (mtDNA) synthesis and expression as well as mtDNA packaging (Asin-Cayuela and Gustafsson, 2007; Ekstrand et al., 2004; Kaufman et al., 2007; Kukat et al., 2011; Larsson et al., 1998). In animal models, a *TFAM* knockout in mice severely depletes mtDNA, abolishes oxidative phosphorylation and leads to embryonic lethality (Larsson et al., 1998). A heart-specific knockout results in cardiomyopathy during embryogenesis and neonatal death (Li et al., 2000). By contrast, TFAM overproduction in transgenic mice increases mtDNA content (Ekstrand et al., 2004; Larsson et al., 1998) and also ameliorates cardiac failure (Ikeuchi et al., 2005), neurodegeneration, and age-dependent deficits in brain function (Hokari et al., 2010). TFAM is the most abundant component of mitochondrial nucleoids, which are protein complexes associated with mtDNA that orchestrate genome replication, expression, and inheritance (Bogenhagen et al., 2008, 2012; Kukat et al., 2011). The in vivo packaging of mtDNA by TFAM has been estimated at ~35–50 (Cotney et al., 2007; Maniura-Weber et al., 2004) to ~1,000–1,700 molecules per genome (Ekstrand et al., 2004; Kanki et al., 2004; Kaufman et al., 2007; Kukat et al., 2011; Pellegrini and Scorrano, 2007). Higher TFAM:mtDNA ratios are interpreted as resulting in tighter compaction of mtDNA and reduced accessibility to transcription, replication, or repair factors, whereas lower ratios are predicted to permit increased accessibility. Recently, a debate has emerged as to whether TFAM is required for basal transcription, and whether it functions as both an activator and a repressor of transcription (Asin-Cayuela and Gustafsson, 2007; Falkenberg et al., 2002; Litonin et al., 2010; Lodeiro et al., 2012; Shi et al., 2012; Shutt et al., 2010; Sologub et al., 2009; Zollo et al., 2012). Further experiments are required to resolve this debate. Another fundamental question that has yet to be addressed pertains to the regulatory processes that control the binding and release cycle of TFAM at the mitochondrial genome.

Mitochondrial Lon belongs to the AAA+ family of proteins (ATPases associated with various cellular activities) and requires ATP hydrolysis to degrade proteins (Venkatesh et al., 2012). As a quality-control protease, human Lon selectively eliminates certain abnormal proteins (Bota and Davies, 2002). However, Lon also degrades some folded (Ondrovicová et al., 2005) and regulatory proteins (Granot et al., 2007; Tian et al., 2011). Although the majority of Lon is soluble within the matrix, it is also present in mitochondrial nucleoids (Bogenhagen et al., 2008). Lon binds mtDNA in a sequence-specific and strand-specific manner, showing low-affinity binding to single-stranded sequences on the heavy-strand that forms parallel G-quartets

(Chen et al., 2008; Liu et al., 2004). In cultured mammalian cells, Lon preferentially binds to the control region of mtDNA (Lu et al., 2007), which contains the heavy-strand promoter (HSP) and light-strand promoter (LSP) (Bonawitz et al., 2006; Falkenberg et al., 2007), as well as an origin of replication. Lon is thus uniquely poised at the mitochondrial genome to regulate mtDNA metabolism or to remodel nucleoid composition. In *Drosophila* cells with normal mtDNA content, the knockdown of Lon increases the levels of both TFAM protein and mtDNA, whereas overexpression of Lon decreases these levels (Matsushima et al., 2010). By contrast, in human cells with normal mtDNA content, changes in Lon expression do not alter TFAM or mtDNA levels (Lu et al., 2007) (Figures S1A–S1C available online). Such differences between flies and humans may be linked to phylogenetic diversity in the structure and metabolism of mtDNA. Interestingly, in human tissue or cells that are depleted of mtDNA, the protein levels of TFAM are dramatically reduced, even though transcript levels are the same as in control cells with mtDNA (Larsson et al., 1994; Seidel-Rogol and Shadel, 2002). These findings implicate Lon in the proteolytic turnover of TFAM in humans as well as in flies.

Here, we demonstrate that PKA-mediated phosphorylation of TFAM within high-mobility-group box 1 (HMG1) occurs inside the mitochondrion, resulting in rapid and selective degradation by the Lon protease. HMG1 phosphorylation of TFAM leads to DNA dissociation and reduced transcriptional activation. We propose that phosphorylation of TFAM within HMG1 causes electrostatic repulsion of the DNA phosphate backbone, thereby providing a mechanism for regulating mtDNA binding and release, which are essential for the maintenance and expression of the mitochondrial genome.

RESULTS

DNA-Bound TFAM Is Resistant to Lon-Mediated Proteolysis

We set out to test the hypothesis that Lon selectively degrades TFAM that is not bound to mtDNA. Purified TFAM and Lon (Figure S1D) were incubated with or without ATP/Mg^{2+}. TFAM was rapidly degraded by Lon only when ATP was present (Figure 1A). TFAM was not degraded by ClpXP, which is another AAA^+ protease in the mitochondrial matrix (Figures S1D–S1F). To determine whether DNA binding by TFAM affected proteolysis, single-stranded or double-stranded DNA (ssDNA or dsDNA, respectively) oligonucleotides were preincubated with TFAM prior to addition of Lon (Figure 1B); a higher Lon concentration was added to accelerate proteolysis and to accentuate the effect of DNA. We employed ssDNAs corresponding to heavy- and light-strand sequences upstream of LSP (LSP^{HS} and LSP^{LS}, respectively), as well as a dsDNA corresponding to the TFAM binding site ($dsDNA^{TFAM}$) (Figure 1B) (Dairaghi et al., 1995). DNAs with greater relative binding to TFAM conferred greater resistance to Lon (Figures 1B–1E). TFAM was strongly stabilized by LSP^{HS} and $dsDNA^{TFAM}$, but only marginally by LSP^{LS} (Figure 1B). Correspondingly, LSP^{HS} and $dsDNA^{TFAM}$ showed greater relative binding to TFAM compared to LSP^{LS} in gel shift and Southwestern assays (Figures 1C–1E). LSP^{HS} migrates as a fast-mobility linear ssDNA and as a slow-mobility G-quartet species on native gels (Figure 1C) (Chen et al., 2008; Liu et al., 2004).

Since Lon is a ssDNA-binding protein (Chen et al., 2008; Liu et al., 2004), its binding to LSP^{HS} or $dsDNA^{TFAM}$ may directly block its protease activity, resulting in TFAM stabilization. However, neither LSP^{HS} nor $dsDNA^{TFAM}$ inhibited Lon-mediated degradation of casein (Figure 1F). Although Lon shows the greatest relative affinity for LSP^{HS}, as compared to other mtDNA sequences (Chen et al., 2008; Liu et al., 2004), its binding to LSP^{HS} is substantially weaker than binding of TFAM to LSP^{HS} (unpublished results). Thus, it is not possible to test whether DNA-bound Lon degrades DNA-free TFAM.

Downregulation of Lon in Cells with Severe mtDNA Deficits Blocks TFAM Degradation and Increases mtDNA Content

The link between TFAM levels, mtDNA copy number, and Lon-mediated proteolysis was investigated in HeLa cells with either normal mtDNA content or severe mtDNA deficits. TFAM was strongly detected in cells with normal mtDNA levels (ρ^+ cells) (Figure 2A). By contrast, TFAM was barely detected in cells that were irreversibly depleted of mtDNA (ρ^0 cells) (Figure 2A). ρ^0 cells were generated by culturing ρ^+ cells with ethidium bromide for an extended period to deplete mtDNA, and then selecting the cells that were auxotrophic for pyruvate and uridine (King and Attardi, 1996). TFAM levels were also strikingly reduced in cells with low mtDNA content (ρ^{low} cells) (Figure 2A), which were generated by ethidium bromide incubation for a shorter time, resulting in cells that did not exhibit pyruvate and uridine auxotrophy. Although ρ^{low} cells had severely reduced mtDNA levels similar to ρ^0 cells (Figure S2), they still retained residual copies of the genome (see Figure 2C). To determine whether the reduced TFAM levels in ρ^{low} cells resulted from Lon-mediated proteolysis, we genetically knocked down Lon. A substantial increase in TFAM was observed upon transient siRNA knockdown of Lon, whereas TFAM levels remained unchanged in cells transfected with control siRNA (Figure 2B). By contrast, no upregulation of TFAM was observed when ClpP was knocked down (Figure 2B).

One potential consequence of upregulating TFAM by knocking down Lon in ρ^{low} cells is a coordinated increase in mtDNA. Lentiviral delivery of shRNAs targeting Lon was employed, as this approach showed higher efficiency, less toxicity, and lower background compared to transfections with siRNA or shRNA plasmids (data not shown). At 1 week, mtDNA copy number was essentially the same in control and Lon knockdown ρ^{low} cells. However, by ~2 weeks, the Lon depletion led to increased mtDNA copy number, which was reproducibly observed compared to the control (Figure 2C, 13 days). During this time period, Lon knockdown cells showed reduced levels of Lon and upregulated levels of TFAM (Figure 2D, 13, 17, and 27 days). By ~3 weeks, mtDNA copy number had declined in Lon knockdown cells (Figure 2C, 23 days), even though Lon was still depleted and TFAM remained elevated during this period (Figure 2D, 27 days). We speculate that the lentivirus-mediated knockdown of Lon in ρ^{low} cells only transiently increases mtDNA content even though TFAM remains elevated, because Lon is needed for mitochondrial homeostasis and genome

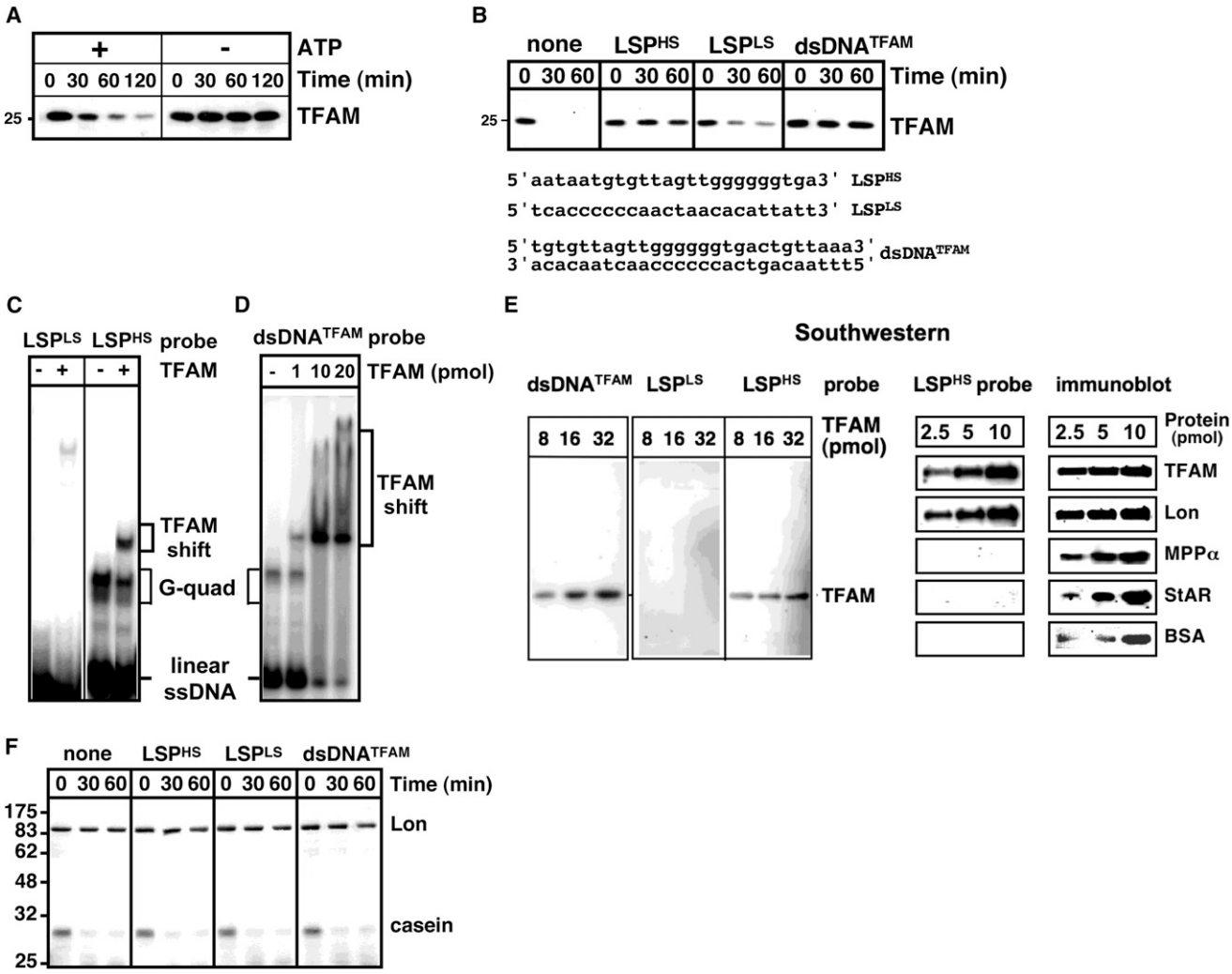

Figure 1. TFAM Bound to DNA Is Resistant to Lon

(A and B) TFAM (80 nM) was incubated with Lon (50 nM) with or without ATP (2 mM) (A), or preincubated with DNA oligonucleotides (4 μM) for 10 min on ice prior to adding Lon (80 nM) and ATP (2 mM) (B); TFAM was detected by immunoblotting.

(C–E) TFAM (1 pmol or as indicated) incubated with radiolabeled DNA (4 pmol) was analyzed by gel shift (C and D) or Southwestern (E) assays. Southwestern membranes were probed with radiolabeled DNA or immunoblotted for TFAM, Lon, the mitochondrial processing peptidase α subunit (MPPα), steroidogenic acute regulatory protein (StAR), and bovine serum albumin (BSA).

(F) Lon (66 nM) was preincubated with or without DNA (4 μM) for 10 min before adding casein (3 μM) and ATP, and was analyzed by SDS-PAGE and Coomassie Blue staining. See Figure S1.

maintenance in HeLa ρ^{low} cells. Further experiments are required to address this issue.

Bortezomib Inhibits Lon and Blocks TFAM Degradation

TFAM degradation was also explored by pharmacologically inactivating Lon. Previous work shows that Lon is selectively inhibited by MG132 and MG262 but not by epoxomicin, although all three are inhibitors of the 20S proteasome (Frase and Lee, 2007; Granot et al., 2007). We demonstrated that Lon is also inhibited by the MG262-related compound bortezomib (Velcade, PS-341) (Figure 3A), which is used to treat multiple myeloma and mantle cell lymphoma (Adams, 2004). Bortezomib inhibited Lon-mediated cleavage of a dipeptide substrate with an IC_{50} value of 17 nM, which was comparable to its inhibition of the 20S proteasome with an IC_{50} value of 2.3 nM (Figures 3A and 3B). Bortezomib (0.6–10 μM) also blocked the turnover of TFAM in ρ^{low} cells at concentrations that inhibited the proteasome-mediated degradation of p53, which is constitutively unstable in HeLa cells (Figure 3C) (Scheffner et al., 1990). Bortezomib, like MG262, selectively stabilized TFAM in ρ^{low} cells, whereas epoxomicin did not (Figure 3D). By contrast, all three inhibitors stabilized p53 (Figure 3D). Although bortezomib stabilized TFAM in both ρ^{low} and ρ^{0} cells, no change was observed in ρ^{+} cells (Figure 3E), most likely because the vast majority of TFAM was bound to mtDNA and was Lon-resistant.

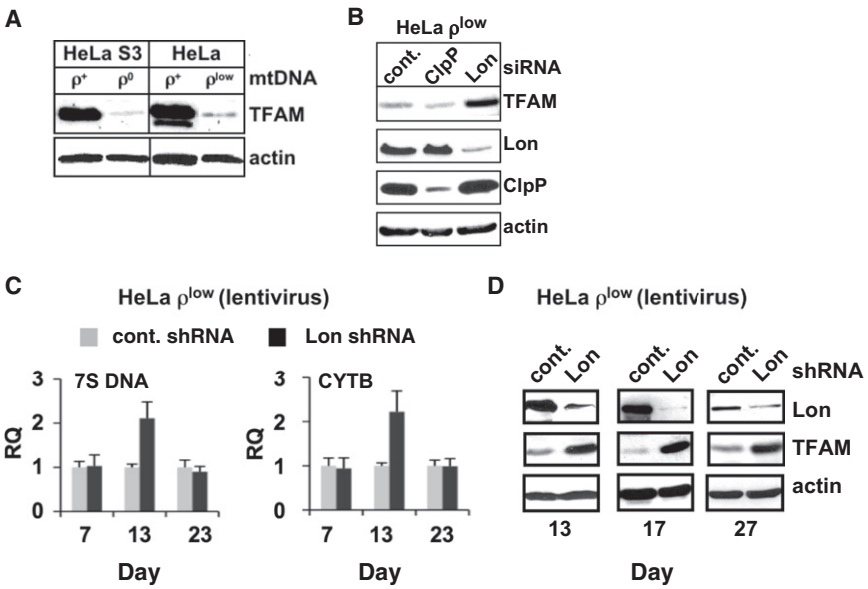

Figure 2. Lon Knockdown in mtDNA-Depleted Cells Increases TFAM and mtDNA

(A) Extracts from HeLa ρ^+, ρ^0,and ρ^{low} cells were blotted for TFAM. Overexposure permits detection of TFAM in ρ^0 and ρ^{low} cells. A lower-molecular-weight TFAM band in ρ^+ cells is likely a processed form or breakdown product.

(B) Extracts from ρ^{low} cells transfected with control, Lon or ClpP siRNAs were blotted for TFAM, Lon, ClpP, and actin.

(C) Total DNA was isolated from ρ^{low} cells transduced with control or Lon shRNA lentivirus (moi 5) and relative quantitation (RQ) of mtDNA was determined by quantitative PCR of 7S DNA and the *CYTB* gene using the nuclear *APP* gene as an endogenous control. Data represent at least three independent experiments. Error bars indicate ±SEM.

(D) Extracts from ρ^{low} cells transduced as in (C) were immunoblotted for Lon, TFAM, and actin. See Figure S2.

TFAM Carrying Inactivating HMG Box Mutations Is Degraded by Lon

Using HeLa ρ^+ cells with normal mtDNA levels, we tested whether the failure of TFAM to bind DNA led to degradation by Lon. A TFAM mutant was engineered with inactivating mutations in HMG1 and 2, in which lysines 51, 52, and 156, as well as arginine 157, were replaced by alanines or glycines (Figure 4A, $TFAM^{HMG1/2}$). The crystal structure of TFAM bound to LSP dsDNA (Ngo et al., 2011; Rubio-Cosials et al., 2011) predicts that these HMG box substitutions will reduce DNA-binding affinity by disrupting hydrogen-bond formation with DNA (see Figure 4D). When corresponding mutations are introduced in the *S. cerevisiae* Abf2p, which shows sequence homology to TFAM, impaired DNA binding is observed (Zelenaya-Troitskaya et al., 1998).

We examined the stability of $TFAM^{HMG1/2}$ and wild-type TFAM transiently expressed in ρ^+ cells that were genetically or pharmacologically downregulated for Lon using RNA interference (RNAi) or bortezomib, respectively (Figures 4B and 4C). TFAM degradation was examined using a cycloheximide (CHX) chase to block cytosolic protein synthesis over a time course. Cell extracts from each time point were immunoblotted with antibodies recognizing the myc-tag fused to mutant or wild-type TFAM, distinguishing overexpressed from endogenous TFAM. In the absence of CHX (T = 0), $TFAM^{HMG1/2}$ and wild-type TFAM were detected as two molecular-weight forms (Figures 4B and 4C): the cytosolic full-length precursor protein carrying a mitochondrial targeting sequence, and the mature processed protein lacking a targeting sequence. When normal levels of Lon were present ($control^{RNAi}$), both the precursor and mature forms of $TFAM^{HMG1/2}$ rapidly declined during the chase, with half-lives of <1 hr (Figure 4B). However, when Lon was knocked down (Lon^{RNAi}), both the precursor and mature forms of $TFAM^{HMG1/2}$ were stabilized, suggesting that Lon degraded both the partially translocated precursor and the mature form (Figure 4B). By contrast, the knockdown of Lon did not alter the half-lives of either the wild-type precursor or the mature protein (Figure 4B). Little conversion of the wild-type precursor to the mature form was observed, most likely because the precursor was expressed at high levels, and not degraded, thus saturating the import channel. Bortezomib addition during the chase stabilized mature $TFAM^{HMG1/2}$ with little effect on the precursor protein (Figure 4C). Like the Lon knockdown, bortezomib treatment had virtually no effect on the levels of the mature or processed wild-type TFAM in ρ^+ cells (Figures 4B and 4C).

In Vitro Phosphorylation of TFAM by PKA

We surmised that TFAM binding to mtDNA might be antagonized by HMG phosphorylation, leading to Lon-mediated degradation. As PKA activity has been shown in the mitochondrial matrix (Acin-Perez et al., 2011; Agnes et al., 2010; Robin et al., 2003; Schmidt et al., 2011), we tested whether TFAM was in vitro phosphorylated by the catalytic subunit of PKA. In the presence of γ^{32}-ATP, TFAM was radiolabeled (Figure S3A). Liquid chromatography tandem mass spectrometry (LC-MS/MS) identified four in vitro phosphorylated sites within TFAM—serines 55, 56, and 61 in HMG1, and serine 160 in HMG2 (Figure S3B). The phosphorylation of HMG1 serines 55, 56, and 61 is noteworthy, as these serines interact with DNA in the X-ray structures of TFAM bound to LSP DNA (Figure 4D) (Ngo et al., 2011; Rubio-Cosials et al., 2011). The yeast homolog of TFAM- Abf2p is also in vitro phosphorylated by PKA within HMG1 and exhibits impaired DNA binding (Cho et al., 2001). However, Abf2p phosphorylation does not lead to proteolysis, and its mechanistic significance is unclear. In vitro studies show that Abf2p weakly stimulates transcription 1.5- to 2-fold (Parisi et al., 1993), in contrast to TFAM, which stimulates transcription >1,000-fold (Litonin et al., 2010). Abf2p, like TFAM, has a common role in mtDNA packaging and inheritance (Zelenaya-Troitskaya et al., 1998).

Phosphorylation of TFAM by PKA within Mitochondria

To investigate whether TFAM phosphorylation occurs in cells and in mitochondria, we purified the overexpressed protein

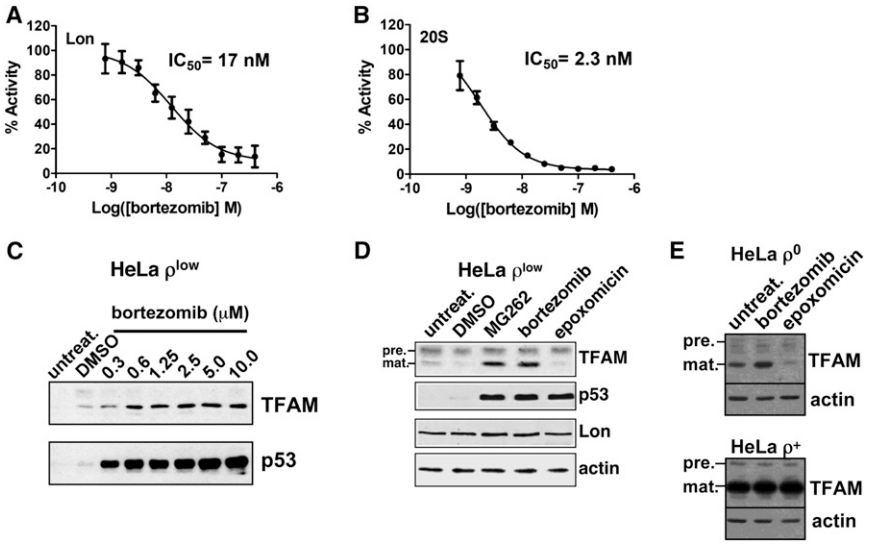

Figure 3. Lon-Dependent Proteolysis of TFAM Is Blocked by Bortezomib and MG262 but not Epoxomicin

(A and B) Lon (200 nM monomer) (A) or 20S (3 nM complex) (B) peptidase activities were measured using the fluorescent dipeptide substrate AA_2-Rh110 (6 μM) incubated in the presence or absence of bortezomib at 37°C for 3 hr. Fluorescence was normalized to the percent activity of no-drug control. Results represent at least three independent experiments. Error bars indicate ±SEM.

(C) ρ^{low} cells were incubated with or without bortezomib for 18 hr and extracts were blotted for TFAM or p53.

(D and E) ρ^{low}, ρ^0, or ρ^+ cells were treated with DMSO, bortezomib (5 μM), MG262 (1.25 μM), or epoxomicin (1 μM) for 18 hr; extracts were blotted for TFAM, p53, Lon, or actin. TFAM precursor (pre.) and mature (mat.) proteins are indicated.

from HEK293 cells treated with or without MG262 to inhibit Lon. Phosphopeptide enrichment and LC-MS/MS-identified phosphorylated sites within TFAM (Figure S3C). Peptides phosphorylated at serines 55 and/or 56 were detected only when TFAM was isolated from MG262-treated cells (Figure S3C). Based on this finding, we produced anti-phosphopeptide antibodies to these sites. An anti-phosphoserine 55 ($pTFAM^{ser55}$) antibody was comprehensively validated, and this antibody specifically recognized both the phosphopeptide and in vitro phosphorylated TFAM, as well as wild-type phosphoTFAM expressed in cells; however, it did not recognize $TFAM^{SSAA}$, in which serines 55/56 were replaced by alanines (Figure S3D). Using this antibody, we showed that endogenous $pTFAM^{ser55}$ was present in cell and mitochondrial extracts only when Lon was genetically or pharmacologically downregulated (Figures 4E, 4F, 4I, and S3E). The PKA inhibitors H89 or KT5720 blocked the appearance of $pTFAM^{ser55}$ in Lon-inhibited cells (Figures 4F and 4I), implicating PKA as the kinase that mediates this phosphorylation.

PKA-catalyzed phosphorylation occurs both inside and outside mitochondria (Acin-Perez et al., 2011; Agnes et al., 2010; Robin et al., 2003; Schmidt et al., 2011). A complete PKA system that is present within the mitochondrial matrix is activated by cAMP generated by a soluble adenylyl cyclase (Acin-Perez et al., 2009). However, the signals targeting PKA to the matrix and the specific catalytic and regulatory isoforms functioning in the matrix are unknown. To discriminate whether $TFAM^{ser55}$ was phosphorylated in the cytosol or in the mitochondrial matrix, TFAM was coexpressed with either the PKA catalytic α subunit (PKAα) or a mitochondrial targeted PKAα mutant (mtsPKAα). PKAα was diffusely distributed in the cytosol, whereas mtsPKAα colocalized with mitochondria (Figure 4G). When cytosolic PKAα and TFAM were coexpressed, no increase in $pTFAM^{ser55}$ was observed in either the presence or absence of MG262 (Figure 4H). The activity of PKAα was confirmed by its phosphorylation of histone deacetylase 8 (HDAC8) at serine 39 (Lee et al., 2004) (Figure S3F). By contrast, mtsPKAα coexpression led to the appearance of $pTFAM^{ser55}$, which accumulated upon Lon inhibition by MG262 (Figure 4H). The PKA inhibitors H89 or KT5720 blocked phosphorylation of overexpressed TFAM at serine 55 (Figure 4I). We showed that $pTFAM^{ser55}$, mtsPKAα, and Lon were present in isolated mitochondria that had been trypsin-digested to remove copurifying nonmitochondrial proteins, as well as the cytosolic domains of outer-membrane proteins (Figure S3G). Thus, $pTFAM^{ser55}$ and mtsPKAα were protected within mitochondria. To determine whether TFAM was phosphorylated within mitochondria, in organello reactions were performed by incubating trypsin-treated mitochondria expressing mtsPKAα with [γ-^{32}P]ATP (Figures 4J and S3G). We showed that phosphorylated radiolabeled TFAM was immunoprecipitated from mitochondria (Figure 4J). Collectively, these results demonstrated that phosphorylation of $TFAM^{ser55}$ occurs in mitochondria and not in the cytosol.

TFAM Phosphomimics within HMG1, but Not HMG2, Are Degraded by Lon

We predicted that TFAM phosphomimics unable to bind mtDNA would be degraded by Lon and would be selectively stabilized by bortezomib or MG262, but only nominally by epoxomicin (see Figure 3D). TFAM phosphomimics carrying serine-to-aspartate substitutions within HMG1 or 2 were expressed in ρ^+ cells (Figures 5A and 5B). In untreated HeLa ρ^+ cells, wild-type TFAM was detected as both precursor and mature proteins (Figure 5B). MG262, bortezomib, and epoxomicin increased both precursor and mature forms of TFAM, suggesting that proteasome inhibition stabilized the precursor, leading to increased import and increased levels of mature protein. A markedly different inhibitor response profile was observed for the phosphomimic $TFAM^{SSDD}$ in which serines 55 and 56 in HMG1 were replaced by aspartates. In untreated cells, the precursor of $TFAM^{SSDD}$ was strongly detected, but the mature protein was virtually absent (Figure 5B). The Lon-inhibiting compounds MG262 and bortezomib substantially increased mature $TFAM^{SSDD}$, whereas only a marginal increase was observed with epoxomicin. All three inhibitors slightly increased precursor

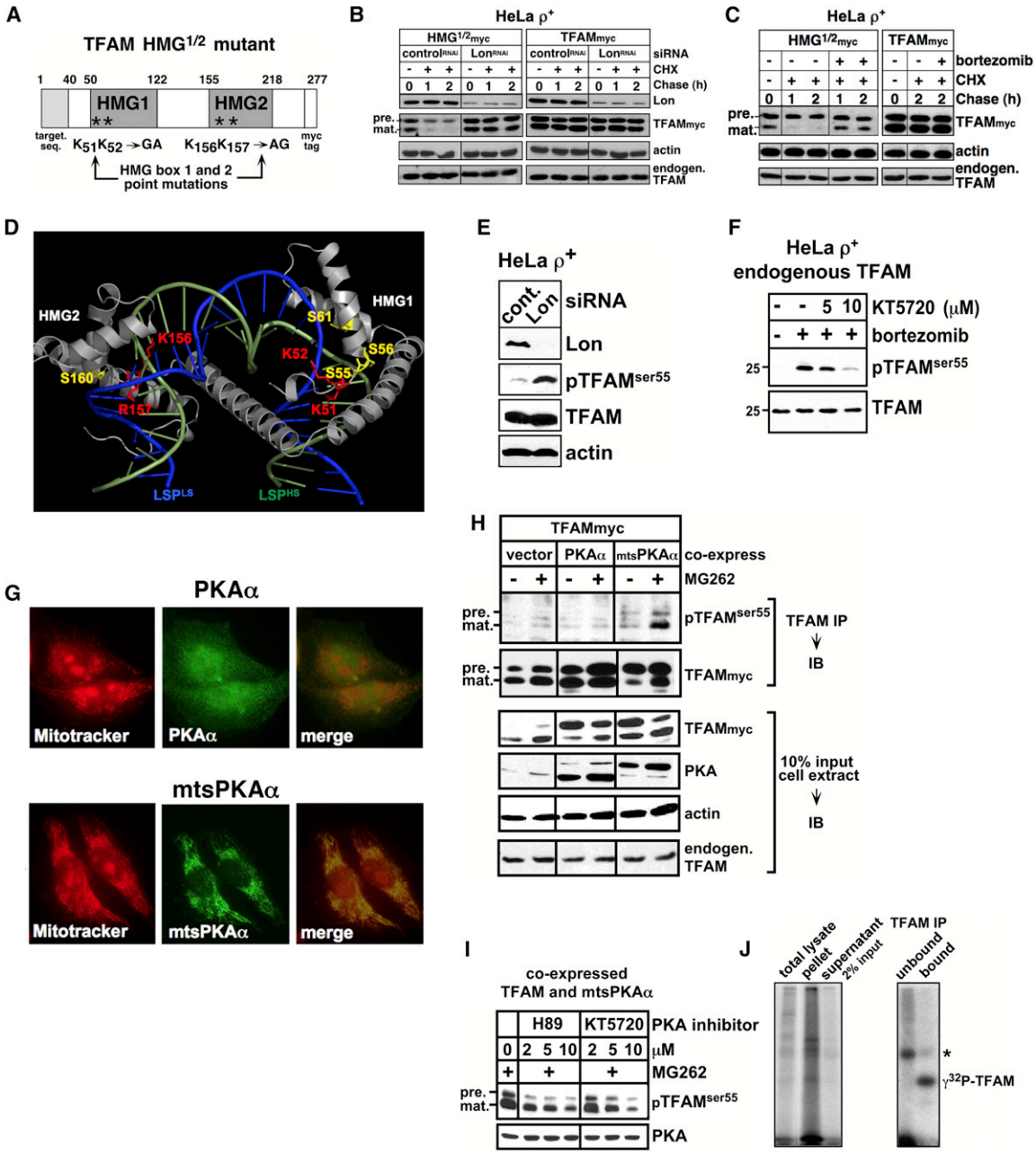

Figure 4. HMG Box Mutation or PKA-Dependent Phosphorylation of TFAM Leads to Degradation by Lon

(A) Diagram of HMG box mutants of TFAM.

(B) HeLa ρ^+ cells were transfected twice with siRNAs on Days 0 and 2; on Day 3, the cells were transfected with plasmids for expressing $HMG^{1/2}{}_{myc}$ or $TFAM_{myc}$; and on Day 4, cells were chased with cycloheximide (CHX, 100 μg/ml). Extracts were blotted for TFAM, Lon, and actin.

(C) ρ^+ cells were transfected as in (B), chased with CHX and bortezomib (5 μM), and blotted as in (B).

(D) TFAM-DNA complex (Protein Data Bank ID code 3TMM) showing HMG box lysines 51, 52, and 156, and arginine 157 (red); HMG box serines 55, 56, 61, and 160 (yellow); LSP^{LS} (blue); and LSP^{HS} (green).

(E and F) Endogenous $pTFAM^{ser55}$ in ρ^+ cells knocked down for Lon for 48 hr (E), or treated with or without the PKA inhibitor KT5720 or bortezomib (5 μM) for 16 hr (F).

(G) Fluorescent double labeling of Mitotracker Orange and overexpressed PKAα or mtsPKAα in ρ^+ cells.

(H) $TFAM_{myc}$ coexpressed with mtsPKAα or PKAα in ρ^+ cells treated with or without MG262 (1.25 μM) for 16 hr. Anti-TFAM immunoprecipitates were blotted for $pTFAM^{ser55}$, TFAM, and PKA.

(I) $TFAM_{myc}$ and mtsPKAα were coexpressed in ρ^+ cells and treated with MG262 (1.25 μM) in the presence or absence of H89 or KT5720 for 16 hr.

(J) Trypsin-treated mitochondria coexpressing TFAM and mtsPKAα were incubated with [γ-^{32}P]ATP, then lysed (total lysate) and centrifuged (resulting in supernatant and pellet). The supernatant was immunoprecipitated for TFAM (bound and unbound). Samples were analyzed by SDS-PAGE and autoradiography. *Autophosphorylated PKAα coimmunoprecipitated with TFAM (Figures S3A and S4H). See also Figure S3.

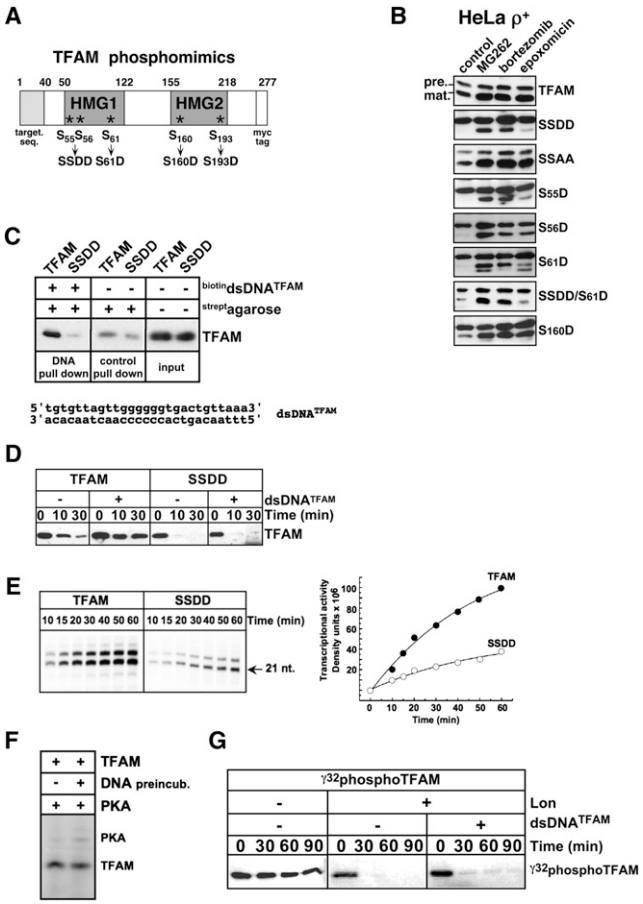

Figure 5. TFAM Phosphorylation Modulates DNA Binding, Transcriptional Activation and Lon Sensitivity

(A) Diagram of TFAM phosphomimic mutants at serines phosphorylated by PKA in vitro.

(B) ρ^+ cells transiently expressing wild-type TFAM or phosphomimics were treated with DMSO, MG262 (1.25 μM), bortezomib (5 μM), or epoxomicin (1 μM); extracts were blotted with anti-myc antibodies. Actin controls are shown in Figure S4A.

(C) TFAM or TFAM^{SSDD} was preincubated with or without biotinylated dsDNA^{TFAM} prior to incubation with streptavidin agarose. Pull-down and protein input were blotted for TFAM.

(D) TFAM or TFAM^{SSDD} (160 nM) was preincubated with or without dsDNA^{TFAM} (8 μM), before adding Lon (70 nM) and ATP (2 mM); reactions were blotted for TFAM.

(E) Transcription reactions with TFAM or TFAM^{SSDD} using the LSP promoter template with α-[^{32}P]ATP.

(F) TFAM (160 nM) was preincubated with a 300-fold molar excess of DNA prior to adding PKA (2,500 U) and [γ-^{32}P]ATP (8 μCi) at 30°C for 2 hr.

(G) [γ-^{32}P]-labeled PKA phosphorylated TFAM was preincubated with or without dsDNA^{TFAM} before adding Lon, as in (D), and visualized by autoradiography. See also Figures S3 and S4.

levels of TFAM^{SSDD}. The instability of TFAM^{SSDD} was caused by aspartate substitutions in HMG1, as the alanine-substituted TFAM^{SSAA} was stable and resembled wild-type TFAM (Figure 5B). The single, double, or triple serine-to-aspartate phosphomimics at serines 55, 56, and 61 were also barely detected in untreated controls and were selectively stabilized by the inhibitors to varying degrees (Figures 5B and Figure S4A). TFAM^{S55D} and TFAM^{SSDD} showed the most clear and reproducible upregulation by MG262 or bortezomib, with little effect of epoxomicin (Figure 5B). By contrast, the HMG2 phosphomimics TFAM^{S160D} (Figure 5B) and TFAM^{S193D} (Figure S4A) were not Lon sensitive, as their inhibitor response patterns were similar to wild-type TFAM.

TFAM Phosphorylation at HMG1 Impairs DNA Binding and Transcription and Promotes Lon-Mediated Degradation

We directly tested the ability of TFAM^{SSDD} to bind DNA, which is required for transcriptional activation and resistance to Lon. Purified TFAM^{SSDD} and wild-type TFAM were incubated with biotinylated dsDNA^{TFAM} carrying the TFAM binding site. TFAM^{SSDD} was not significantly pulled down, in contrast to wild-type TFAM (Figure 5C). Because of this DNA-binding defect, preincubating TFAM^{SSDD} with dsDNA^{TFAM} (without biotin) failed to confer Lon resistance, whereas wild-type TFAM was fully stabilized (Figure 5D). To analyze the effect of HMG1 phosphorylation at serines 55 and 56, we compared the transcriptional activation of TFAM^{SSDD} with that of wild-type TFAM. mtDNA transcription is catalyzed by mitochondrial RNA polymerase (mtRNAP), which requires TFAM, as well as another initiation factor, TFB2M, for promoter binding and melting. Together, TFAM and TFB2M operate synergistically to boost the efficiency of mtRNAP (Litonin et al., 2010). We employed a reconstituted transcription system consisting of mtRNAP, TFB2M, and either TFAM^{SSDD} or wild-type TFAM, which were incubated with a DNA template carrying the LSP promoter that drives the synthesis of a 21- to 22-nucleotide transcript (Falkenberg et al., 2002; Sologub et al., 2009). In transcription run-off experiments, TFAM^{SSDD} showed a 3- to 4-fold reduction in activation from LSP, as compared to wild-type TFAM (Figure 5E). Similar results were observed using a DNA template carrying the heavy-strand promoter 1 (HSP1) (Figure S4B). These results suggest that phosphoserines 55 and 56 within the HMG1 prevent DNA binding, attenuate transcriptional activation, and promote Lon-mediated degradation.

Biochemical experiments were carried out to test directly whether TFAM already bound to DNA can be phosphorylated by PKA, and whether PKA-phosphorylated TFAM is degraded by Lon. TFAM was preincubated with and without an ~300-fold molar excess of dsDNA^{TFAM} before adding PKA and γ^{32}-ATP. DNA-bound TFAM was phosphorylated and radiolabeled by PKA to nearly the same extent as DNA-free TFAM (Figure 5F). We further showed that γ^{32}phosphoTFAM was degraded by Lon, and was not stabilized by adding DNA (Figure 5G). Taken together, these results suggest that HMG1 phosphorylation of TFAM affects DNA binding and release, thereby modulating transcriptional activation and sensitivity to Lon-mediated proteolysis.

DISCUSSION

This study reveals mechanisms for rapidly regulating TFAM binding to mtDNA by phosphorylation and proteolysis. We propose that PKA-mediated phosphorylation of TFAM within

HMG1 causes electrostatic repulsion of the DNA phosphate backbone, thus providing a mechanism for regulating mtDNA binding and release. Results of TFAM-DNA binding experiments, along with the crystal structure of the TFAM-LSP complex, suggest a mechanism by which TFAM binds to and bends DNA (Gangelhoff et al., 2009; Ngo et al., 2011; Wong et al., 2009). According to this mechanism, the high-affinity HMG1 domain of TFAM binds LSP first, and subsequent conformational changes in TFAM and mtDNA allow for binding by HMG2, which exhibits lower affinity and less specificity for LSP. TFAM binding to nonpromoter regions of mtDNA likely depends primarily on HMG1. Thus, for promoter and nonpromoter binding, phosphorylation of serine residues within HMG1 will play a major role in affecting the DNA-binding status of TFAM and degradation by Lon.

Mechanisms for dissociating TFAM from mtDNA are critically important, as it may extensively coat the genome, potentially binding every ~20 base pairs (Kukat et al., 2011; Takamatsu et al., 2002). The coating of mtDNA by TFAM mediates the tight compaction of the genome, which affects its replication, transcription, and maintenance. The process of uncoating mtDNA has not been elucidated, but it likely involves the selective and processive dissociation of TFAM. One can envisage that phosphorylation-stimulated release of TFAM from mtDNA decompacts the genome, and that its degradation by Lon permits sustained decompaction. However, HMG1-phosphorylated TFAM may rebind to mtDNA if it is dephosphorylated by phosphatases prior to degradation. Alternatively, unphosphorylated and DNA-free TFAM may be sequestered within nucleoids and protected from Lon proteolysis until it is utilized for genome coating. In addition, newly imported TFAM may bind mtDNA during or soon after translocation and folding of the HMG1 domain, which is ~10 amino acids away from the mature amino terminus. The coordination of phosphorylation/dephosphorylation with proteolysis of TFAM can thus fine-tune its functions in mitochondrial maintenance, expression, and inheritance.

The pathways activating PKA-mediated phosphorylation of TFAM are unknown. Previous work suggests that mitochondrial protein phosphorylation by PKA may be activated by changes in CO_2 and O_2 tension (e.g., ischemia, hypoxia) (Acin-Perez et al., 2009, 2011; Agnes et al., 2010; Papa et al., 2008; Prabu et al., 2006; Sardanelli et al., 2006). PKA within mitochondria is activated by cAMP, which is generated by a matrix-localized soluble adenylyl cyclase that responds to metabolically generated CO_2/HCO_3^- (Acin-Perez et al., 2009; Agnes et al., 2010; Sardanelli et al., 2006). Studies also show that hypoxia and/or ischemia lead to PKA-mediated phosphorylation and functional changes in Complexes I and IV (Acin-Perez et al., 2011; Papa et al., 2008; Prabu et al., 2006). Interestingly, hypoxia and ischemia lead to upregulation of Lon expression, which enables cells to adapt to these stresses (Bota and Davies, 2001; Fukuda et al., 2007; Hori et al., 2002). Further studies are required to determine whether phosphorylation of TFAM is coordinated with that of Complex I or IV subunits.

We speculate that TFAM may be phosphorylated by PKA and possibly other kinases not only in the mitochondrion but also in the cytosol (Figure 6). In this study we show that TFAM is phosphorylated at HMG1 serines 55 and 56 by PKA inside mitochondria and that HMG1 phosphorylation blocks DNA binding and promotes rapid degradation of TFAM by Lon (Figures 4E–4J, 6A, and S3). Both the precursor and mature forms of TFAM are phosphorylated within HMG1 (Figures 4H and 4I). In addition, our proteomics data show that TFAM isolated from HEK293 cells is also phosphorylated at sites outside HMG1, which are not linked to Lon-mediated proteolysis (unpublished data). We envisage that phosphorylation of TFAM alters its activity in diverse ways. Mitochondrial phosphorylation of TFAM at sites outside HMG1 may influence interactions with proteins involved in transcription or nucleoid dynamics (Figure 6B), whereas in the cytosol, phosphorylation of TFAM may alter its degradation by the proteasome (Figure 6C) or its association with the mitochondrial protein translocation machinery (Figure 6D). The phosphorylation of TFAM at single sites or concurrently at multiple sites may provide temporal and spatial cues that coordinate its function and abundance within mitochondria and at mtDNA.

Our finding that the anticancer agent bortezomib inhibits Lon as well as the 20S proteasome raises the question as to whether Lon inhibition is therapeutically beneficial in treating hematological malignancies. Recently, we showed that knocking down Lon in a mantle cell lymphoma cell line leads to cell death (Bernstein et al., 2012). Cancer cells, unlike normal cells, survive a myriad of chronic stresses, such as mitotic, metabolic, oxidative, and proteotoxic stress, that are linked to oncogenesis (Solimini et al., 2007). Previous work shows that Lon supports cell viability during hypoxia, proteotoxicity, and endoplasmic reticulum stress (Bota and Davies, 2001; Fukuda et al., 2007; Hori et al., 2002). Further experiments are required to address the question of whether inhibiting Lon is toxic for certain cancers. In addition, Lon inhibition may have unique therapeutic effects in cancers with mtDNA mutations or reduced mtDNA copy number, which have been linked to increased aggressiveness of cancer cells and resistance to chemotherapeutic agents (Chandra and Singh, 2011; Guo et al., 2011; Yu, 2011). Collectively, the results and perspectives presented here provide a gateway to exploring new pathways in mitochondrial metabolism that depend on TFAM and Lon.

EXPERIMENTAL PROCEDURES

Details regarding the materials, antibodies, cell culture experiments, protein purification, biochemical assays, molecular biology, mitochondrial isolation, and standard methods are provided in the Supplemental Information.

Cells and Cell Culture

HeLa ρ^+, ρ^0, and ρ^{low} cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 units/ml of penicillin streptomycin. ρ^0 and ρ^{low} cells were supplemented with sodium pyruvate (110 μg/ml) and uridine (50 μg/ml).

Plasmid or siRNA Transfection and Lentivirus Transduction

Wild-type and mutant TFAM, as well as PKAα with or without the mitochondrial targeting sequence of cytochrome *c* oxidase subunit 8A, were myc-tagged on the carboxyl terminal. Transfections were carried out using Lipofectamine2000 (Invitrogen), following the manufacturer's procedures. Transductions were carried out at a multiplicity of infection (moi) of 5 following the manufacturer's procedures.

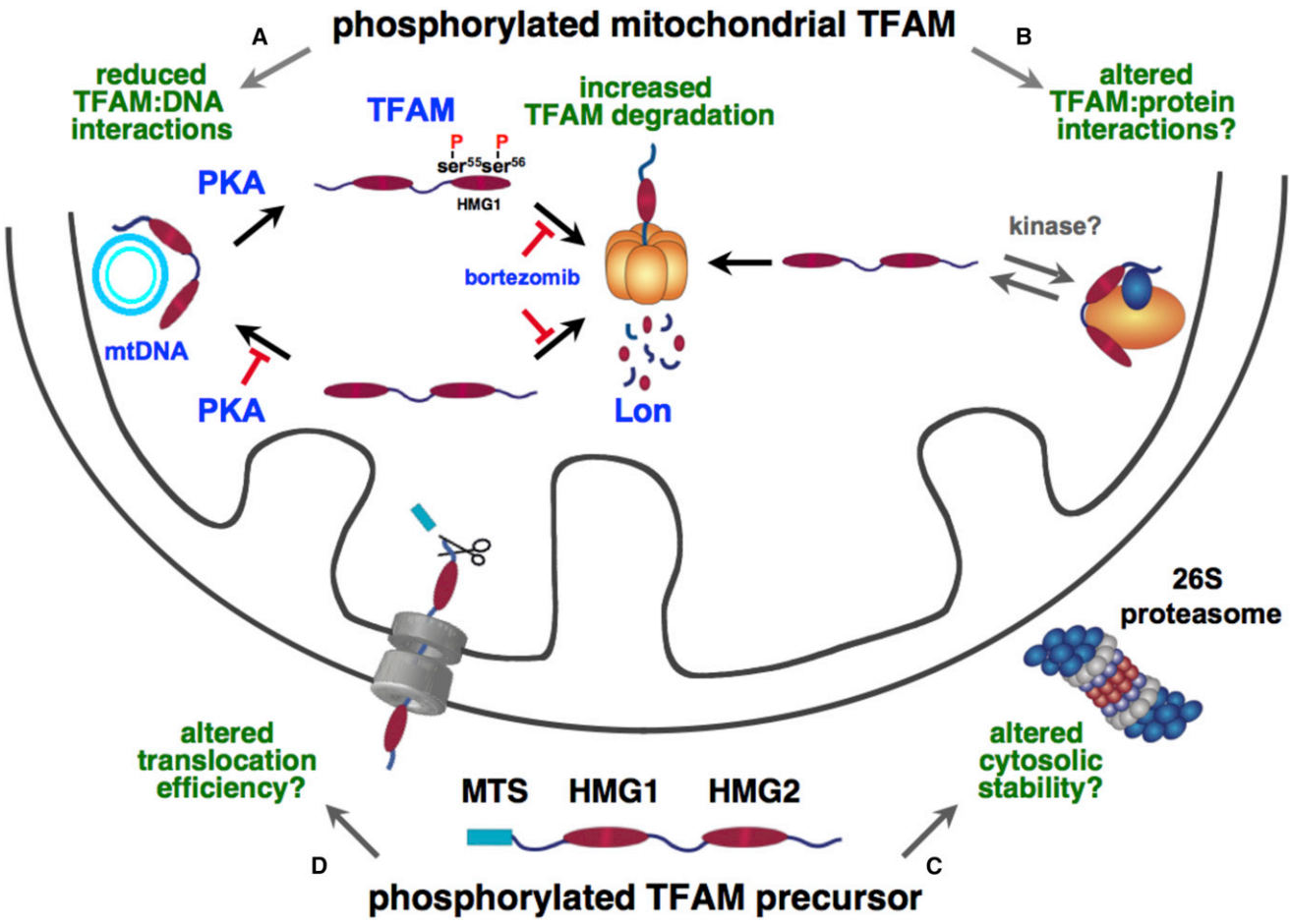

Figure 6. Phosphorylation Potentially Regulates Multiple Aspects of TFAM Biogenesis and Activity

(A and B) Mitochondrial phosphorylation of TFAM. Phosphorylation of TFAM at HMG1 serines 55 and 56 by PKA regulates mtDNA binding and release (A). Phosphorylation of TFAM by PKA or other protein kinases may alter its interactions with other proteins (B).

(C and D) Cytosolic phosphorylation of TFAM precursor. Precursor phosphorylation may alter its degradation by the proteasome (C) or its binding to the protein translocation machinery (D).

Protein Purification

Recombinant human TFAM, Lon, ClpX, and ClpP (Figure S1D) lacked their respective predicted mitochondrial targeting sequences. Lon and TFAM carried an amino-terminal his-tag, and ClpX and ClpP carried a carboxyl-terminal his-tag. Recombinant proteins were purified as described (Bernstein et al., 2012; Litonin et al., 2010; Liu et al., 2004).

Peptidase and Protease Assays

Peptidase activities of recombinant human Lon or the 20S proteasome (Boston Biochem) were measured using rhodamine 110, bis-(CBZ-L-alanyl-L-alanine amide; Anaspec), as described (Bernstein et al., 2012). For protease assays, Lon or ClpXP was incubated with TFAM or casein with or without 2 mM ATP. Reactions were analyzed by immunoblotting or Coomassie Blue staining.

Gel-Shift, Southwestern and DNA Pull-Down Assays

Detailed procedures are described in the Supplemental Experimental Procedures. Gel-shift reactions with TFAM and [γ-^{32}P]-labeled DNA probes were performed as described (Liu et al., 2004). For Southwestern assays, proteins were separated by SDS-PAGE and transferred to nitrocellulose, which was either immunoblotted or probed with [γ-^{32}P]-labeled DNA. For pull-down experiments, TFAM or TFAM^{SSDD} was incubated with biotinylated dsDNA^{TFAM} and then pulled down with streptavidin agarose and analyzed by immunoblotting.

Quantitative PCR

Quantitative PCR was performed with Taqman primers and probes using total DNA isolated from cells (Lu et al., 2007) (see Supplemental Experimental Procedures). Statistical significance was evaluated with Student's unpaired t test.

Reconstituted Mitochondrial Transcription Assay

Transcription reactions were carried out as described (Litonin et al., 2010) (Supplemental Experimental Procedures), using mutant or wild-type TFAM (100 nM), TFB2M (150 nM), and mtRNAP (150 nM).

Phosphorylation Site Identification within Recombinant PKA-Phosphorylated TFAM and Purified Cellular TFAM

Phosphopeptide enrichment was performed using recombinant TFAM that was PKA-phosphorylated or cellular TFAM purified from HEK293 cells (Novoprotein). Phosphorylation-site identification was performed using LC-MS/MS (Center for Advanced Proteomics, New Jersey Medical School). Details of the procedures are described in the Supplemental Experimental Procedures and Figures S3A–S3C.

Immunofluorescence

Double labeling of PKAα or mtsPKAα using anti-myc antibodies and Mitotracker Orange was performed as described (Lu et al., 2003).

TFAM Phosphorylation in Trypsin-Treated Mitochondria

Mitochondria were incubated in buffer with trypsin for 10 min on ice, and then in trypsin inhibitor for 5 min on ice. Trypsin-treated mitochondria were washed and incubated in buffer with [γ-^{32}P]ATP. See Supplemental Experimental Procedures for details of the protocol.

TFAM Immunoprecipitation

HeLa ρ^+ cells or mitochondria were lysed in buffer with protease and phosphatase inhibitors. Cell extracts were immunoprecipitated, as described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.10.023.

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