A legacy role for DNA binding of Lon protects against genotoxic stress

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Abstract:

The quality control protease Lon was originally characterized as a DNA binding protein, yet the physiological consequences of this interaction are not understood. Here we use the α-proteobacteria *Caulobacter crescentus* to show that DNA binding of Lon is critical for DNA damage tolerance. *In vitro*, DNA can directly activate or inhibit Lon activity and promotes degradation of DNA bound proteins by neighboring bound Lon. Bacteria expressing a DNA-binding deficient Lon variant are phenotypically wildtype with respect to normal growth and response to proteotoxic stresses, but are sensitive to genotoxic stresses. Disrupting Lon binding to mitochondria genomes also results in sensitivity to DNA damage but otherwise maintained normal mitochondrial function, consistent with the bacterial ancestry of this organelle. We propose that clearance of overly persistent proteins from DNA, including DNA-protein crosslinks, by the Lon protease is an important damage response that originated in free-living α-proteobacteria and has been preserved during the endosymbiotic transition to mitochondria.

One Sentence Summary: DNA binding by the Lon protease protects against genotoxic damage in a manner that has been preserved from bacteria to mitochondria.

Main Text:

Caulobacter crescentus (*Caulobacter*) is a freshwater dimorphic a-proteobacteria that undergoes a developmental transition during its cell cycle (*1*). Energy dependent proteolysis by AAA+ proteases drives this cell cycle (*2*). For example, the ordered degradation of cell cycle regulators by the ClpXP protease requires the hierarchical assembly of adaptors (*3-9*). In *Caulobacter*, the Lon protease degrades the epigenetic regulator CcrM(*10*), the transcriptional regulator SciP(*11*), and the replication initiator DnaA(*12*). In *E. coli*, Lon is responsible for degrading misfolded proteins as part of quality control (*13, 14*), but one of the original phenotypes of *lon* was sensitivity to DNA damage, exemplified by Evelyn Witkin's original studies using the naturally *lon* deficient B strain to define the genetic basis of radiation resistance (*15*). Subsequent suppressor studies revealed that degradation of the *E. coli* cell division inhibitor SulA by Lon is responsible for a large part of this sensitivity (*16, 17*). Whether Lon plays a role in DNA damage in other bacteria, such as *Caulobacter*, remains poorly understood. In mitochondria, Lon has been clearly implicated in managing the response to protein misfolding stresses although it is unclear how it might affect mtDNA stress (*18-20*). We sought to address these questions beginning from a biochemical perspective.

Although Lon has long been known to bind DNA (*21, 22*), there are conflicting reports on whether DNA acts in an inhibitory or activating role (*23-25*). Using a FITC-casein degradation assay and purified *Caulobacter* Lon protease, we found that DNA clearly modulates Lon activity, but that the outcome of DNA binding is not easily predicted. Genomic DNA isolated from *Caulobacter* inhibits Lon activity, while short dsDNA oligonucleotides or PCR products elicited a more minor effect (Figure 1). By far, the most dramatic variation was seen with single stranded DNA oligonucleotides: some activate Lon ~2-fold and others repress activity >5-fold (Figure 1). Because casein is not

known to bind DNA, our data suggests that DNA can both directly activate or inhibit general Lon protease activity. Analyses of these oligonucleotides did not show any strong correlation between activation/inhibition and specific sequences, length, dinucleotide motifs, or T_m (Figure S1). However, there was a strong correlation in the strength of either inhibition or activation with the predicted ability to form G-quadruplex (G4) structures (Figure 1,S1).

G4 structures are noncanonical DNA structures with unknown biological functions, but are associated with changes in transcription, replication and DNA damage (*26*). Interestingly, G4 favoring sequences have been previously associated with binding to human mitochondrial Lon (*20, 27*), but the consequence of this interaction on Lon activity was not determined. To validate this correlation with G4 structures, we focused on a strongly inhibitory oligonucleotide (OPC498) which contains a predicted hairpin structure and G4-favoring region. Mutating the hairpin structure did not affect the inhibitory activity (Figure 1) nor binding to Lon (Figure S1), but eliminating the G4 region resulted in loss of inhibition and Lon binding (Figure 1, S1). A G4-containing sequence known to bind the human mitochondrial Lon (*20*) also inhibits protease activity (Figure 1). Our conclusion is that while Lon can bind many DNA sequences, it has a particularly affinity for guanine rich sequences that can form G4 structures.

One proposed role for DNA binding of Lon is that it could promote degradation of DNA bound proteins (*28-30*). We tested this hypothesis by using DnaA, a known *in vivo* substrate of Lon that is poorly degraded *in vitro* in the absence of Lon regulators (*12*). In *Caulobacter,* DnaA binds to origin DNA most strongly through G-box motifs (*31*). We used a dsDNA containing the first origin G-box (G1box) and the natural downstream guanine-rich region that we predicted would bind Lon. As expected, Lon alone degrades

DnaA poorly, but addition of the G1box dsDNA dramatically accelerates degradation (Figure 1). Both the features are important as mutation of the G-box or removal of the guanine-rich region eliminate the enhanced degradation (Figure 1). We hypothesized the G1box dsDNA is acting as a simple scaffold and tethers DnaA with Lon to drive proteolysis. If so, then excess scaffold should counterintuitively suppress DnaA degradation because the ternary Lon-G1box-DnaA complex will be depleted when pairwise Lon-G1box and DnaA-G1box species accumulate at high scaffold concentrations. Consistent with our model, addition of excess dsDNA initially activates substrate degradation ($K_{activation}$ ~ 300 nM; Supp Info), but inhibits at higher concentration (Figure 1).

In order to explore the specific role of DNA binding in Lon regulation, we required a Lon variant that was incapable of binding DNA, but otherwise active. Based on previous work in *E. coli* Lon (*32*), we mutated a cluster of lysines (K302, K304, K306, K307) to glutamic acids to generate a DNA binding deficient Lon, Lon4E. Purified Lon4E degrades FITC-casein with similar kinetics as wildtype Lon, but is insensitive to inhibition by OPC498 (Figure 2). Δ*lon Caulobacter* exhibit profound morphological defects including cell body elongation, increased stalk organelle length and loss of motility in soft agar (*10*)(Figure 2). Importantly, cells expressing only Lon4E rescue these phenotypes (Figure 2; Figure S2), confirming our *in vitro* results that Lon4E is fully active. Loss of Lon results in increased levels of its substrates DnaA and CcrM (*10, 12*) and Lon4E largely restores steady-state levels to wildtype (Figure 2). Degradation of CcrM is also restored by the Lon4E allele (Figure S2). Finally, RNA-seq experiments show that the Lon4E allele does not dramatically alter the transcriptome while loss of Lon has a significant impact on expression of over a hundred genes (Figure S2). We conclude that

Lon4E complements the function of wildtype Lon during normal, unstressed growth conditions.

We found that in addition to morphological defects, cells lacking Lon were highly sensitive to many stresses (Figure 2). For example, Δ*lon* strains grew poorly on media containing L-canavanine, which induces protein misfolding, and when stressed by a variety of DNA damaging agents (Figure 2). Interestingly, Lon4E strains were as resistant to proteotoxic stress as wildtype based on their response to L-canavanine, but were more sensitive to genotoxic stresses (Figure 2). This difference was most dramatic for those agents that induce crosslinking of DNA such as mitomycin C (MMC) or cisplatin (CIS) which must be repaired with complex multistep pathways such as interstrand crosslink repair and translesion synthesis (*33*). However, minor differences were also present for compounds such as methyl methanosulfonate (MMS), which results in damage that can be resolved by simpler pathways such as base excision or mismatch repair (*33*). Clearly, loss of Lon results in the most dramatic phenotypes in all stress conditions (Figure 2), suggesting that DNA binding of Lon has a specific role in responding to DNA damaging conditions, but non DNA-dependent Lon protease activity is crucial for stress tolerance in general.

Mitochondria are theorized to have an endosymbiotic origin (*34*). Specifically, the current model is that a free-living α-proteobacterium was engulfed by the ancestor of the modern eukaryotic cell (*35*). Because *Caulobacter* is an α-proteobacteria, we explored if the need for DNA binding of Lon in tolerating DNA damage was preserved in mitochondria. In *C. elegans*, mitochondrial Lon (LONP-1) is required for development and growth (*19*). Based on homology, we engineered a variant of *lonp-1* with mutations

in the corresponding predicted DNA binding residues to generate LONP-1^{4E} and used CRISPR/Cas9 to produce worms expressing only this allele at the endogenous locus. Importantly, we found that worms expressing only the LONP-1^{4E} variant at endogenous levels were healthy, grew and developed normally (Figure 3). Because LONP-1 has a known role in mitochondrial protein quality control by recognizing and degrading diverse proteins that incur oxidative damage (*18*), we infer that LONP-1^{4E} is capable of maintaining proteostasis during animal development. Pull-down experiments indicated that LONP-1 robustly binds mitochondrial genomes *in vivo* as previously suggested(*25, 29*), but LONP-1^{4E} does not (Figure 3). Therefore, DNA binding of mitochondria Lon is not essential for normal development or growth.

The mitochondrial unfolded protein response (UPR^{mt}) couples the state of mitochondrial protein homeostasis to an upregulation of nuclear encoded chaperone genes. Induction of the UPR^{mt} requires the transcription factor ATFS-1, which is targeted to the nucleus when mitochondria undergo stress. Complete depletion of LONP-1 activates the UPR^{mt}, as determined by upregulation of the transcriptional mitochondrial chaperone reporter *hsp-6$_{pr}$::gfp* (*36*) (Figure 3) consistent with a role for LONP-1 in the maintenance of mitochondrial function (*19*). LONP-1^{4E} worms do not activate the UPR^{mt} suggesting mitochondrial protein homeostasis is intact (Figure 3). However, LONP-1^{4E} worms exposed to cisplatin showed a dramatic activation of UPR^{mt} that is ATFS-1 dependent while wildtype worms show no response (Figure 3). Importantly, mitochondrial dysfunction driven by depletion of the protein quality control protease SPG-7 caused UPR^{mt} activation in both wildtype and LONP-1^{4E} animals to the same degree (Figure 3), as does exposure to paraquat which induces mitochondrial oxidative stress (Figure 3). These results suggest that similar to what is seen in *Caulobacter*, loss of DNA binding of Lon in mitochondria results in a specific intolerance to genotoxic stress.

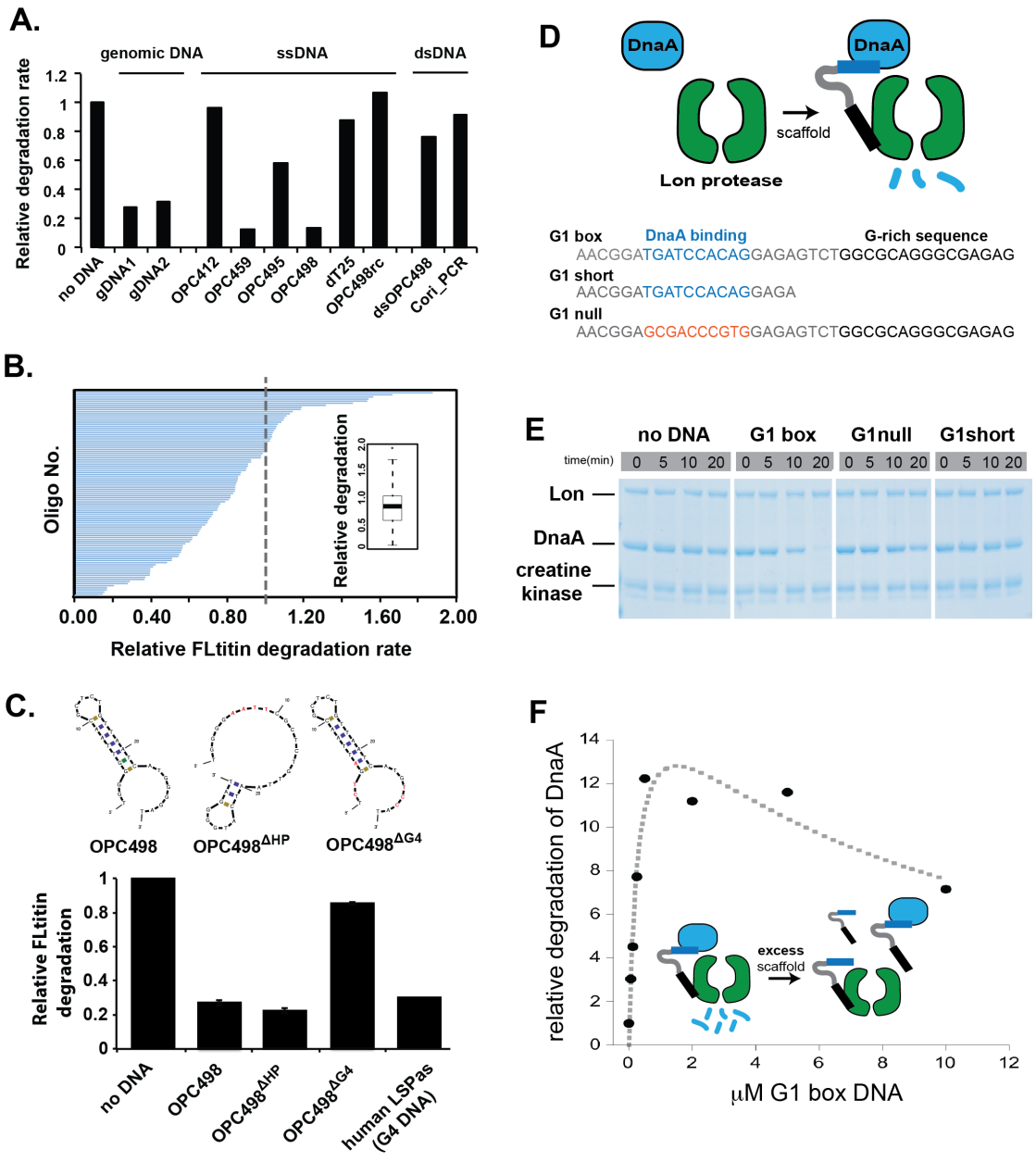
In other work, we had used transposon sequencing in *Caulobacter* to explore Lon-dependent regulatory pathways and identified genes wherein insertions are synthetically lethal with loss of Lon. One of these genes is *uvrD* (Figure 4), a helicase originally known for its role in DNA damage repair (*37*). UvrD has also been shown to be a machine that proficiently evicts proteins bound to DNA, such as disassembling RecA nucleofilaments (*38, 39*) and stalled RNAP complexes (*40*). Based on our biochemical observation that Lon can degrade substrates via a DNA tether (Figure 1) we speculated that Lon could be particularly useful at degrading overly persistent DNA-bound protein complexes. Therefore, loss of Lon would result in accumulation of proteins on DNA that would be lethal for the cell in the absence of other clearance factors such as UvrD. Consistent with this model, we were able delete *uvrD* from wildtype cells, but not from Δ*lon* cells (Figure S4) and depletion of Lon in a Δ*uvrD* strain results in lethality (Figure 4). We could easily delete other DNA damage response genes (*recA, mmcA*, *imuB*) in a Δ*lon* background to generate viable strains that show no epistasis with respect to genotoxic stresses, suggesting that the synthetic lethality between *lon* and *uvrD* is likely due to loss of protein eviction rather than a general consequence of DNA damage intolerance (Figure 4).

Finally, we directly tested if Lon was responsible for clearing persistent protein complexes by evaluating the most extreme persistent case of DNA-protein crosslinks. We used 5-azacytidine, a chemotherapeutic that when incorporated into DNA forms adducts with cytosine methyltransferases resulting in toxic DNA-protein crosslinks (*41*). Lon was critical for survival in the presence of 5-azacytidine and this toxicity was due to DNA-protein crosslink formation as deletion of the cytosine methyltransferase

CCNA_03741(*42*) protects cells lacking Lon from 5-azacytidine (Figure 4). Together, these results support a model where Lon and protein eviction factors (such as UvrD) work together to clear persistent protein complexes, a pathway that is particularly important during complex DNA damage repair.

The role of DNA binding in Lon has been enigmatic since its initial observation (*21, 22*). Previous reports differ on whether DNA binding enhances (*24*), inhibits (*23*), or does nothing (*25*) to Lon protease activity. Our work shows that all outcomes are possible and consistent with earlier work (*20*) we find that Lon is capable of binding many different sequences, but has preference for sequences with propensity to form G4 structures. Given that G4 structures are associated with DNA damage (*26*), this selectivity may aid in recruiting Lon to sites where clearance of proteins is particularly important. Our working model is that Lon binding to DNA allows it to recognize neighboring proteins and serves as an additional layer of quality control to reduce the residence time of persistent complexes (Figure 4). This oversight works in concert with protein displacing factors, such as UvrD, and is particularly crucial for irreversibly persistent complexes such as DNA-protein crosslinks (Figure 4). It is noteworthy that repair of DNA-protein crosslinks in eukaryote nuclear genomes have recently been shown to be dependent on DNA binding proteases (*43*), but no such proteases have been identified in prokaryotes. Our work suggests that Lon can perform this function in bacteria. Finally, we have shown that the same properties of DNA binding in Lon that are important for genotoxic stress responses in *Caulobacter* are also important in mitochondria, consistent with the endosymbiotic α-proteobacteria ancestry of this organelle (*34, 35*). Thus, the legacy of DNA binding in Lon and subsequent protection from genotoxic stress has been preserved through the evolution of the eukaryotes.

Figure 1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ssDNA
dsDNA
Relative degradation rate
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1
0.8
0.6
0.4
0.2
0
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gDNA1
gDNA2
OPC412
OPC459
OPC495
OPC498
dT25
OPC498rc
dsOPC498
Cori_PCR
B.
Oligo No.
Relative degradation
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0.40
0.80
1.20
1.60
2.00
Relative FLtitin degradation rate
C.
OPC498
OPC498ΔHP
OPC498ΔG4
Relative FLtitin degradation
no DNA
OPC498
OPC498ΔHP
OPC498ΔG4
human LSPas (G4 DNA)
D
DnaA
DnaA
scaffold
Lon protease
G1 box
DnaA binding
G-rich sequence
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G1 short
AACGGATGATCCACAGGAGA
G1 null
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E
no DNA
G1 box
G1null
G1short
time(min)
0 5 10 20
0 5 10 20
0 5 10 20
0 5 10 20
Lon
DnaA
creatine kinase
F
relative degradation of DnaA
excess scaffold
μM G1 box DNA

Figure 1. DNA binding affects Lon activity directly and as an adaptor. **A**. Genomic DNA (gDNA), single strand oligonucleotides (ssDNA) or double-stranded annealed oligonucleotides or PCR products (dsDNA) have different effects on Lon degradation of the model substrate casein. **B**. Different oligonucleotide sequences can either activate or inhibit Lon protease activity. **C**. Inhibitory activity of oligonucleotide OPC498 which contains a G tetrad (G4) prone region is dependent on the presence of the G4 region, but not other secondary structures. **D**. Schematic to show DnaA binds specifically to G box containing DNA and dsDNA constructs used to test adaptor activity. **E**. dsDNA capable of binding both DnaA and Lon facilitates rapid degradation of DnaA, which is normally slowly degraded *in vitro* in these conditions. **F**. DnaA degradation was monitored as a function of increasing dsDNA G1 box adaptor. Inset shows prediction of reduced ternary complex formation due to excess scaffold and partial site binding.

Figure 2

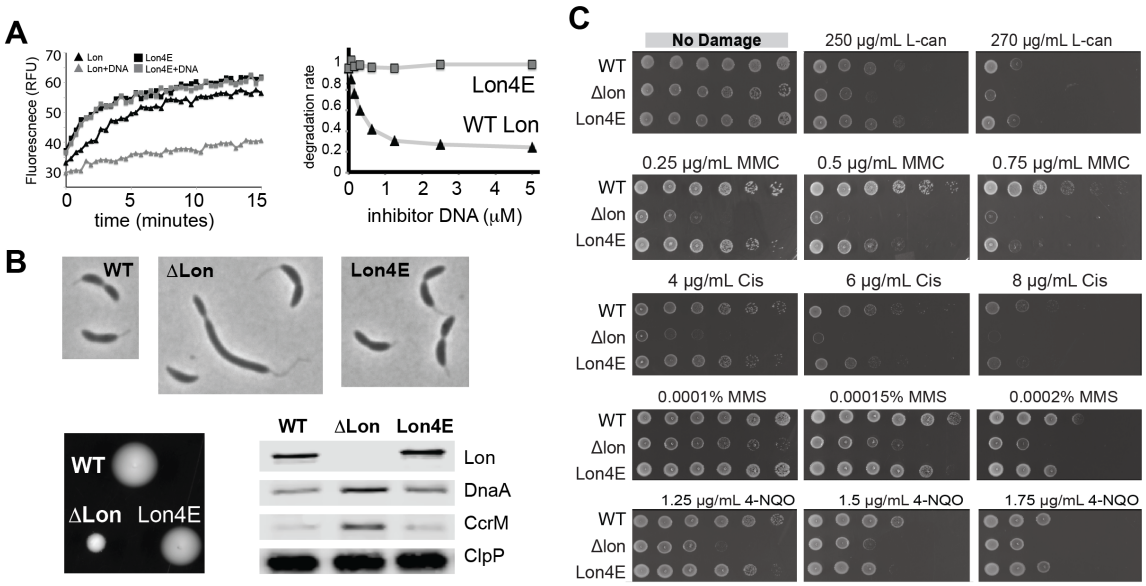
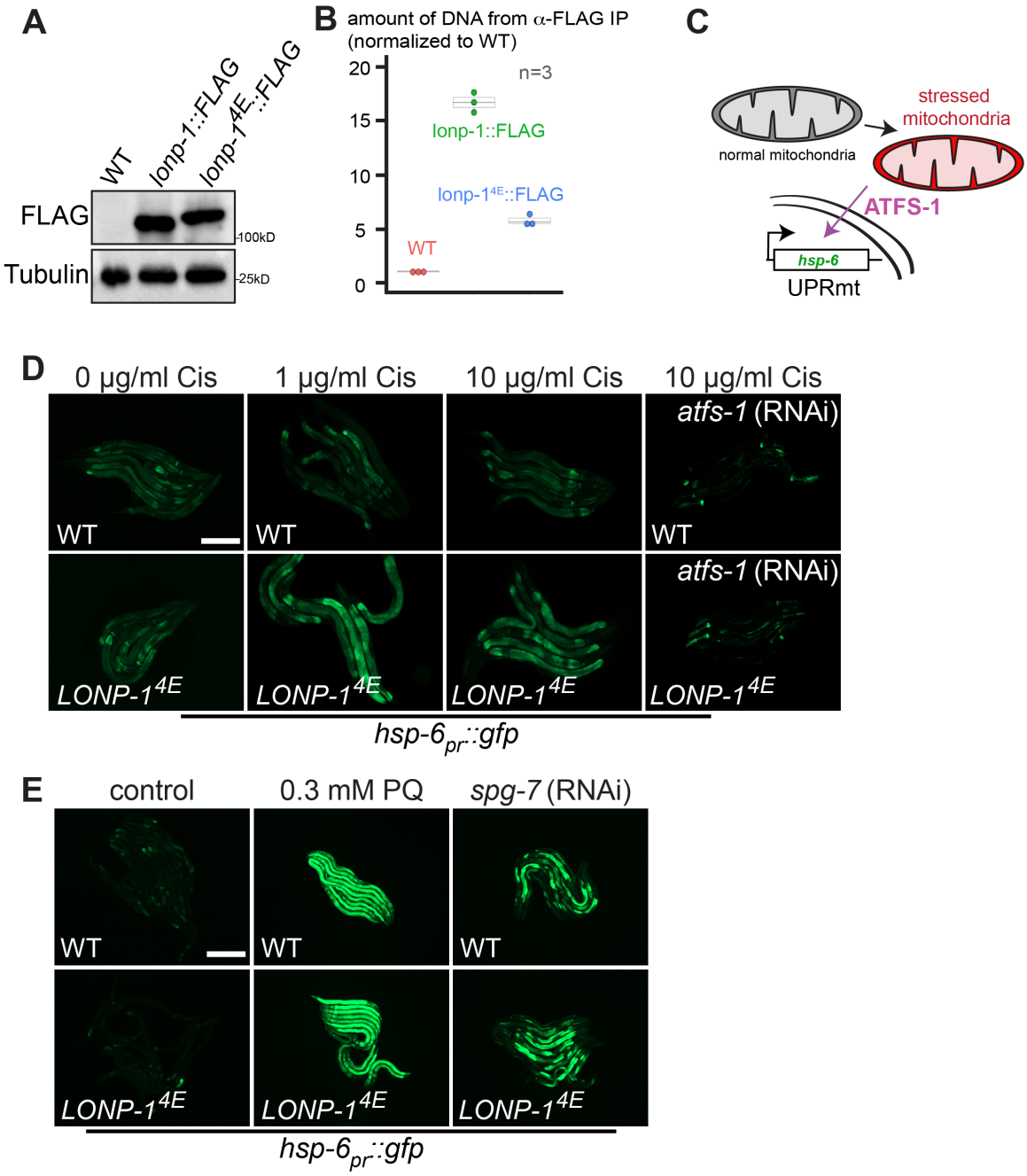


Figure 2. DNA binding mutants of *Caulobacter* Lon rescue normal growth but not DNA damage tolerance. **A**. Lon4E degrades FITC-Casein as well as WT Lon, but fails to respond to DNA. **B**. Morphology, stalk length, and motility defects of cells lacking Lon are not seen in Lon4E strains (see Figure S2 for additional details). Levels of known Lon substrates (CcrM and DnaA) are elevated in Δ*lon* cells (CcrM = 245% +/- 9%; DnaA = 162% +/- 5% of WT; n=3), but mostly reduced to WT levels in the Lon4E mutant (CcrM = 128 +/- 10% of WT, DnaA = 116 +/- 2% of WT; n=3). **C**. Lon4E results in sensitivity to genotoxic stresses. Serial dilutions (10-fold dilutions left to right) of wildtype (WT), Lon deficient (Δ*lon*), or DNA binding deficient Lon expressing (Lon4E) strains on media containing various concentrations of either proteotoxic (L-canavanine (L-can)) or genotoxic (mitomycin C (MMC), cisplatin (Cis), methyl methanosulfonate (MMS), 4-nitroquinolone (4NQO)) compounds.

Figure 3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lonp-1^4E::FLAG
FLAG
Tubulin
100kD
25kD
B
amount of DNA from α-FLAG IP
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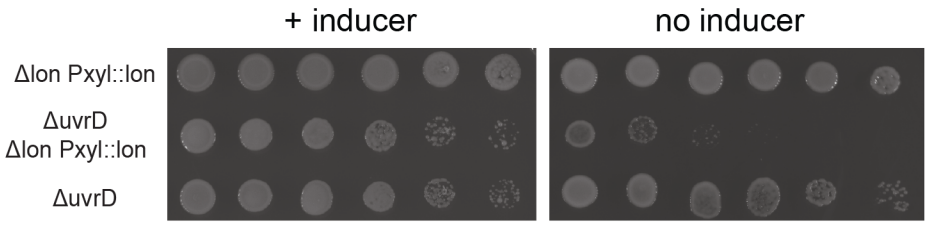
n=3
lonp-1::FLAG
lonp-1^4E::FLAG
WT
20
15
10
5
0
C
stressed
mitochondria
normal mitochondria
ATFS-1
hsp-6
UPRmt
D
0 µg/ml Cis
1 µg/ml Cis
10 µg/ml Cis
10 µg/ml Cis
atfs-1 (RNAi)
WT
LONP-1^4E
hsp-6pr::gfp
E
control
0.3 mM PQ
spg-7 (RNAi)
WT
LONP-1^4E
hsp-6pr::gfp

Figure 3. DNA binding mutants of mitochondria Lon restore normal development but are sensitive to DNA damaging conditions. **A**. Immunoblots of lysates from WT worms, or strains expressing LONP-1-FLAG or LONP-1^{4E}-FLAG. Tubulin serves as a loading control. **B**. ChIP of mtDNA with FLAG antibody in WT, LONP-1-FLAG and LONP-1^{4E}-FLAG strains. **C.** Cartoon illustrating hsp-6 reporter of mitochondria stress. **D.** Photomicrographs of *hsp-6*$_{pr}$*::gfp* WT or LONP-1^{4E} worms raised on DMSO or cisplatin, scale bar, 0.5 mm. **E.** Photomicrographs of *hsp-6*$_{pr}$*::gfp* WT or LONP-1^{4E} worms raised on paraquat (PQ) or *spg-7*(RNAi).

Figure 4

A



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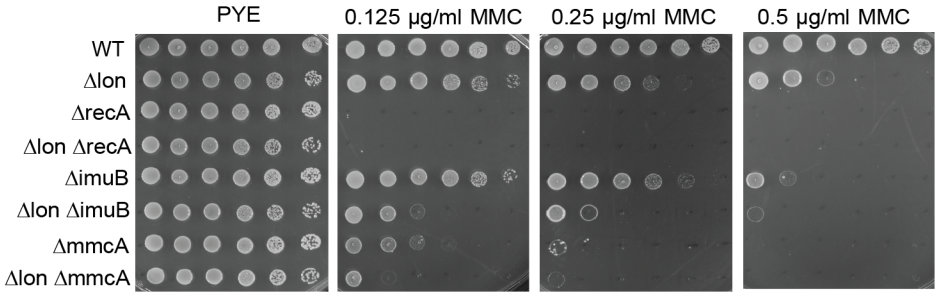

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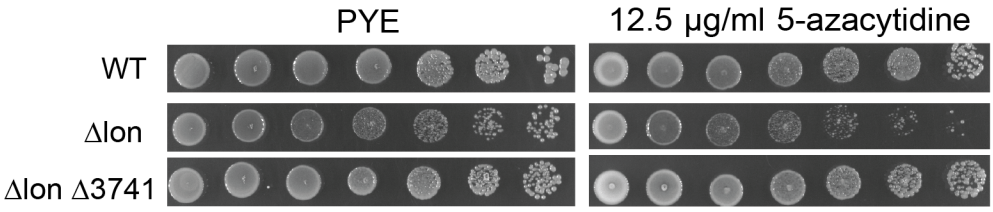

D

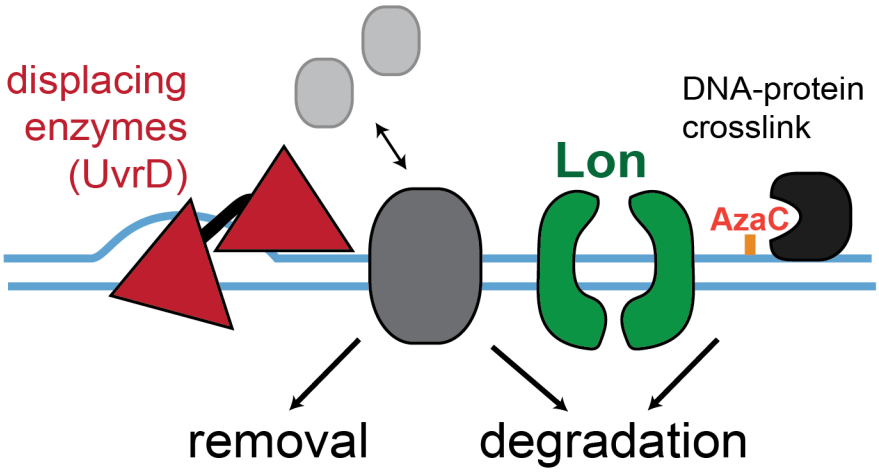

Figure 4. DNA binding of Lon promotes DNA-bound protein clearance. **A**. Depletion of Lon in Δ*uvrD* strains carrying a xylose inducible Lon (Pxyl::Lon) as the sole copy in the cell results in reduced fitness. **B**. *lon* shows no epistasis with other components of DNA damage response such as *recA*, *imuB*, and *mmcA*. **C.** Δ*lon* strains are sensitive to 5-azacytidine, which generates toxic DNA-protein adducts due to crosslinking of cytosine methyltransferases. Deletion of the cytosine methyltransferase CCNA_03741 (Δ3741) in a Δ*lon* background reduces toxicity. **D**. DNA binding proteins dwell on DNA for a specific amount of time, but if they are overly persistent, must be removed by either displacing enzymes, such as UvrD, which remove them through eviction, or Lon can also degrade these complexes. This role is particularly important for degrading covalent DNA-protein crosslinks, such as those generated by 5-azacytidine (AzaC).

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Supplementary Materials:

Materials and Methods

Figures S1-S3

Tables S1-S2

References and Notes

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