Mycobacterial CarD defines a novel mechanism of response to starvation stress

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Abstract

Under starvation conditions, bacteria tend to slow down their translation rate by reducing rRNA synthesis, but the way they accomplish that may vary in different bacteria. In *Mycobacterium* species, transcription of rRNA is activated by the RNA polymerase (RNAP) accessory transcription factor CarD, which interacts directly with RNAP to stabilize the RNAP-promoter open complex formed on rRNA genes. The functions of CarD have been extensively studied, but the mechanisms that control its expression remain obscure. Here, we report that the level of CarD was tightly regulated when mycobacterial cells switched from nutrient-rich to nutrient-deprived conditions. At the translational level, an antisense RNA of *carD* (AscarD) was induced in a SigF-dependent manner to bind with *carD* mRNA and inhibit CarD translation, while at the post-translational level, the residual intracellular CarD was quickly degraded by the Clp protease. AscarD thus worked synergistically with Clp protease to maintain CarD at the minimal level to help mycobacterial cells cope with the nutritional stress. Altogether, our work elucidates the regulation mode of CarD and delineates a new mechanism for the mycobacterial starvation response, which is important for the adaptation and persistence of mycobacterial pathogens in the host environment.

Introduction

Bacterial starvation response refers to the physiological changes occurring in bacteria due to the lack of external nutrients during their growth and reproduction (1). Under starvation conditions, bacterial cells usually reduce the synthesis of rRNA and ribosome proteins (2, 3). The mechanisms of starvation response that have been elucidated in such bacteria as *Escherichia coli* and *Bacillus subtilis* work primarily by reducing rRNA transcription via decreasing the stability of the transcription initiation complex (4, 5).

Mycobacterium is a widespread genus of Gram-positive bacteria that comprises several important pathogens, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis, which kills ~1.5 million people every year. One of the main difficulties in eliminating *M. tuberculosis* is that it usually responds to various host stresses, such as nutritional starvation, low oxygen, and low pH, by entering into a dormant state, which renders the organism extremely resistant to host defenses and antibacterial drugs (6). This genus also includes non-pathogens, such as *M. smegmatis*, which is widely used as a model organism for mycobacterial research. At present, the starvation response mechanisms of mycobacterial cells remain obscure.

Mycobacterial RNA polymerase (RNAP) is usually less efficient in forming RNAP-promoter open complex (RPo) than *E. coli* RNAP on the rRNA genes (7), and the RPo formed is rather unstable and readily reversible (7, 8). To overcome this deficiency, mycobacterial cells have evolved two accessory transcription factors, CarD and RbpA, that help RNAP form a stable RPo (9-11). Both are global transcription factors that interact directly with RNAP to regulate the transcription of many downstream genes, including those of rRNA (9, 12, 13). CarD stabilizes mycobacterial RPo via a two-tiered kinetic mechanism. First, CarD binds to the RNAP-promoter closed complex (RPc) to increase the rate of DNA opening; then, CarD associates with RPo with a high affinity to prevent the DNA bubble collapse (7, 8, 10). Although binding of CarD to RNAP tends to increase the stability of RPo, it may also delay the dissociation of RNAP from the promoter region and thus hinder transcription progress (11). Therefore, CarD may also inhibit the expression of certain genes. Whether CarD activates or inhibits expression of a specific target gene appears to be determined by the kinetics of the initiation complex formation among CarD,

RNAP, and the specific promoter (11, 12). CarD was found to be essential for the survival of mycobacterial cells (14) and weakening the interaction between CarD and RNAP rendered mycobacterial cells more sensitive to oxidative stress, DNA damage, and the effect of some antibiotics (15-17). A recent study showed that CarD regulates (either activates or inhibits) the expression of approximately two-thirds of genes in *M. tuberculosis* (12). Despite the fact that CarD plays such a critical role in mycobacteria, the mechanisms that regulate its cellular levels remain largely uncharacterized.

It is worth noting that CarD was initially thought to inhibit the transcription of rRNA genes, and the transcription of *carD* was up-regulated in response to starvation (14). However, more recently, it was reported that CarD is a transcriptional activator of rRNA genes (8, 18) and the growth rates of mycobacterial cells positively correlate with the CarD content (14, 15, 17). Nevertheless, the expression of CarD is still considered to be up-regulated in response to starvation. If this was the case, the increased CarD would accelerate rRNA synthesis and mycobacterial cells' growth under the starvation condition, which seems to contradict the current consensus (19-21). Therefore, it is important to clarify the regulation of CarD expression under starvation conditions. In the current study, we found that although *carD* transcript levels were upregulated in response to starvation, its protein level dramatically decreased. Further, we found that the reduction of CarD protein level under starvation conditions is a common regulatory mechanism that depends upon functioning of both antisense RNA and Clp protease. Our research describes the mechanisms behind the apparent contradiction between CarD mRNA and protein levels and reveals a new mechanism of mycobacterial response to stress.

Results

CarD protein level increases under genotoxic stresses but dramatically decreases under starvation conditions

CarD is an essential RNAP-interacting protein that regulates the transcription of rRNA genes and many related genes by stabilizing the RPo. While Stallings and colleagues found that the *carD* gene is up-regulated in response to starvation and genotoxic stresses in *M.*

smegmatis strain mc^{2}155 (14), they only monitored the transcriptional level but not the translation of *carD*, which may not truly reflect the CarD protein content. Therefore, to clarify the dynamics of CarD content under the starvation condition and genotoxic stresses, we examined both the *carD* transcript and CarD protein levels in the mc^{2}155 strain by qRT-PCR and Western blot experiments, respectively. As shown in Fig. 1A and B, both *carD* transcript and CarD protein levels increased under genotoxic stresses triggered by H_2O_2, methyl methanesulphonate (MMS), or ciprofloxacin (CIP), which was consistent with the previous reports that CarD may be involved in DNA damage repair (14). However, although the *carD* transcript level increased in response to starvation (Fig. 1A), the CarD protein level, instead, decreased (marked by an arrow in Fig. 1B). This observation indicated that CarD is down-regulated, not up-regulated as previously reported (14), under the starvation condition.

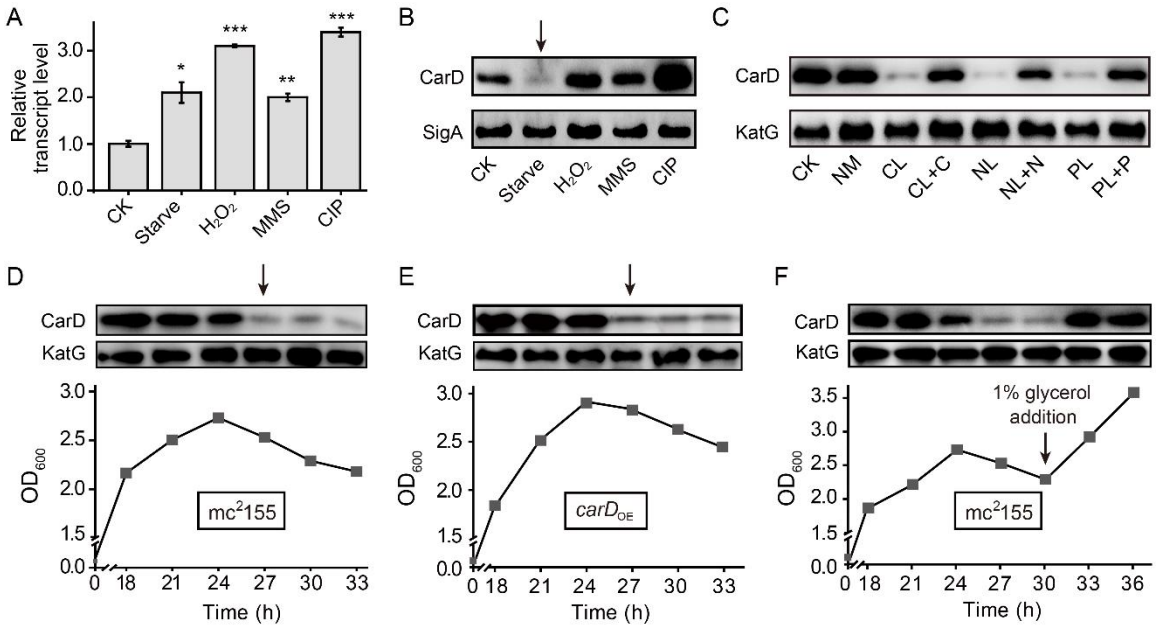

Fig. 1. Changes of CarD transcript and protein levels under starvation and genotoxic stress. A and **B,** the transcript and protein levels of CarD, respectively, under different stress conditions. CK indicates the untreated cells of mc^{2}155. "Starve" means that mc^{2}155 cells were first cultured in the 7H9 medium, and then transferred to phosphate-buffered saline (PBS) for 4 h. For stimulation experiments, 10 mM H_2O_2, 0.1% methyl methanesulphonate (MMS), and 10 mg/mL of ciprofloxacin (CIP) were used. Statistical testing was done using the Student's t-test, with *** indicating p-value <0.001, ** indicating p-value <0.01, and * indicating p-value <0.05. Error bars indicate standard deviation of three biological

replicates. SigA was used as a control in the Western blot experiment. **C,** protein levels of CarD under distinct starvation conditions. CK indicates the untreated exponential cells; NM indicates the exponential cells transferred into the normal medium for 4 h; CL, NL, and PL indicate the exponential cells transferred into carbon-limited, nitrogen-limited, and phosphorus-limited media for 4 h, respectively; CL+C, NL+N, and PL+P indicate the starved mc^2155 cultures supplemented with the corresponding nutrients for 4 h. KatG was used as the control in the Western blot experiments. **D-F,** CarD protein levels at the different growth stages in mc^2155 (**D** and **F**), and *carD* over-expressing strain ($carD_{OE}$, panel **E**). The lower part of the chart shows the respective growth curves with the sampling times.

To investigate whether the decline in the CarD level is due to the lack of a specific nutrient or to a general response to starvation stress, we investigated the changes in CarD levels under carbon-, nitrogen-, and phosphorus-starvation conditions. We first cultured mc^2155 cells to mid-exponential phase (MEP), harvested the cells, and then transferred these cells to the normal medium (NM), carbon-limited (CL), nitrogen-limited (NL), and phosphorus-limited (PL) medium, followed by detecting the respective levels of CarD. The levels of CarD were significantly reduced under all the starvation conditions compared with the CarD level in the normal medium (Fig. 1C). When the nutrient-limited media were supplemented with the corresponding nutrients, CarD returned to normal levels.

Since the mycobacterial cells in the stationary phase are in the state of nutritional starvation (22), we also measured the CarD protein levels at different growth periods of strain mc^2155. As shown in Fig. 1D, the CarD level remained relatively constant in the exponential phase but dropped sharply in the early stationary phase (marked by an arrow in Fig. 1D), which is consistent with the above starvation experiments. To further verify this result, we constructed a *carD* over-expressing strain ($carD_{OE}$) and measured CarD protein levels at different growth periods. Interestingly, despite *carD* overexpression, the CarD protein level still decreased rapidly when the mycobacterial cells entered the stationary phase (marked by an arrow in Fig. 1E). Since the carbon source in the culture medium was likely depleted when the mycobacterial cells entered the stationary phase (22), we speculated that the decrease in the CarD protein level could be caused by carbon

145 starvation. To verify this hypothesis, we added 1% glycerol (glycerol is the main carbon
146 source under normal culture conditions of *M. smegmatis*) to the mc^2155 culture in the
147 stationary phase and measured the CarD protein level 3 and 6 hours after that. As shown
148 in Fig. 1F, the CarD level rapidly increased after the glycerol addition, and the mc^2155
149 cells resumed normal growth. Considering that CarD activates the transcription of rRNA
150 (8, 18), and that cells need to reduce rRNA levels in response to starvation (4), we believe
151 that the reduction in the CarD level under starvation conditions may be an adaptive
152 response of mycobacterial cells. Yet, when nutrients became available, CarD quickly
153 returned to its normal level to allow the cells to resume growth.

154 **CarD levels are dramatically decreased in *M. bovis* BCG and *M. tuberculosis* under**
155 **host-like stress conditions**

156 To investigate whether the rapid reduction of CarD levels under starvation conditions
157 also happens in other mycobacterial species, we carried out starvation experiments in two
158 other mycobacteria, *M. bovis* BCG and *M. tuberculosis* H37Ra. The results are consistent
159 with those in *M. smegmatis*, that is, CarD levels were all significantly reduced in response
160 to carbon-, nitrogen-, and phosphorus-starvation conditions (Fig. 2A, B). When nutrient-
161 limited cultures were supplemented with the corresponding nutrients, CarD returned to the
162 normal levels. In addition, we also measured the CarD levels at different growth phases of
163 the two strains. As shown in Fig. 2C and D, CarD levels were rapidly decreased when BCG
164 and H37Ra cells entered the stationary phase, which is also consistent with the results in
165 *M. smegmatis*. The above results indicate that the rapid reduction of the CarD level in
166 response to starvation is a common phenomenon in mycobacteria, and regulating CarD
167 content to cope with nutritional starvation is a conserved mechanism for the mycobacterial
168 adaptive response.

169 It is important to note that after infecting the host, pathogenic mycobacteria not only
170 suffer from nutritional deprivation but are also exposed to hypoxic and acidic conditions.
171 Therefore, to explore whether CarD plays a role in the adaptation of mycobacterial cells to
172 the host environment, we measured CarD levels under these conditions. As shown in Fig.
173 2E, CarD levels were significantly decreased when the mycobacterial cells were transferred

to the low pH media. For the hypoxic conditions, similarly, CarD levels were also reduced when mycobacterial cells were under hypoxic stress and returned to normal after the cultures were re-aerated. (Fig. 2F and Fig. S1). These results suggest that mycobacterial cells reduce CarD levels in response to host stresses to slow down their translation and metabolic rates, which likely contributes to the adaptation of pathogenic mycobacteria to the hostile environment.

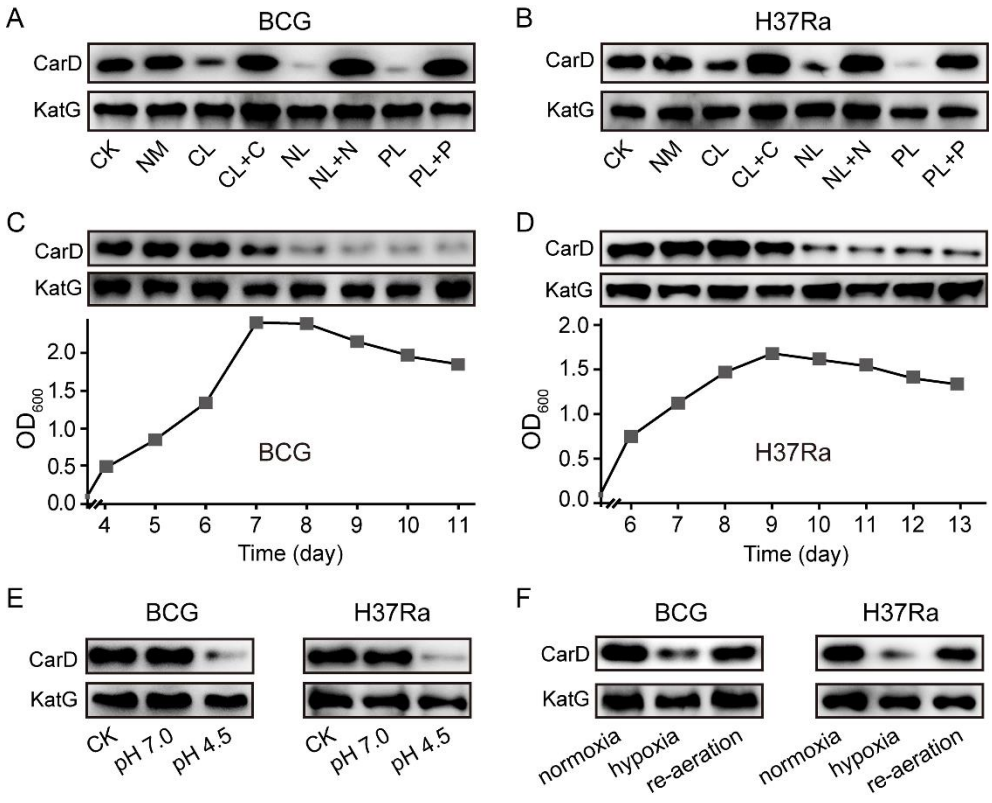

Fig. 2. Changes of CarD levels in *M. bovis* BCG and *M. tuberculosis* H37Ra under host-like stress conditions. A and **B,** the protein levels of CarD in BCG and H37Ra strains, respectively, under distinct starvation conditions. **C** and **D,** CarD protein levels at the different growth stages of BCG and H37Ra, respectively. **E**, CarD protein levels in BCG and H37Ra under different pH conditions. **F**, CarD protein levels in BCG and H37Ra under different oxygen availability conditions.

Clp protease degrades CarD under starvation conditions

Since the CarD level decreased rapidly under nutritional starvation, hypoxic, and acidic conditions, we speculated that CarD might be degraded by a certain protease. To check this

hypothesis, we carried out an *in vitro* CarD degradation experiment. We overexpressed the CarD protein in mc^2155 cells, purified it, and incubated the purified CarD with the total protein extract of mc^2155 cells to check the degradation of CarD. The result showed that CarD protein level decreases substantially when incubated with an untreated total protein extract but no change is observed when CarD is incubated with the boiled protein extract (Fig. S2). This indicates that the reduction of the CarD level under starvation is due to its degradation by a certain protease, which was denatured after boiling and lost the activity to degrade CarD. Moreover, CarD could still be degraded after adding EDTA to the reaction system, suggesting that the CarD-degrading protease was not dependent on metal ions.

Clp protease is a special energy-dependent protease that regulates the response to various stresses. Moreover, previous reports indicated that Clp protease can participate in the rapid degradation of specific proteins under starvation (23, 24). Also, through quantitative proteomics research, Raju et al. found that the CarD protein level in the *clpP*2 conditional deletion mutant was approximately two times higher than in the wild-type strain of *M. tuberculosis* (25). However, that study only measured CarD in the exponential phase, not in the stationary phase. Therefore, it was unclear whether Clp protease mediates the rapid degradation of CarD in the stationary phase. To address this question, we constructed a *clpP*2 conditional mutant (*clpP*2CM) (Methods, and Fig. S3) through the CRISPR/Cpf1-mediated gene editing strategy (26), in which *clpP*2 could be expressed normally only upon addition of 50 ng/mL anhydrotetracycline (ATc), but could not do so when ATc was absent. Then, we measured the CarD protein levels of the *clpP*2CM mutant and the control strain (Ms/pRH2502-*clpP*2, Fig. S3) at different growth stages. The results showed that CarD was rapidly degraded when the control cells entered the stationary phase, regardless of the presence of ATc (Fig. 3A, B). In the *clpP*2CM strain, CarD was also rapidly degraded in the stationary phase when ATc was added to induce the *clpP*2 expression (Fig. 3C) but persisted when *clpP*2 was not induced (Fig. 3D). These results indicate that Clp protease was responsible for the rapid degradation of CarD in the stationary phase.

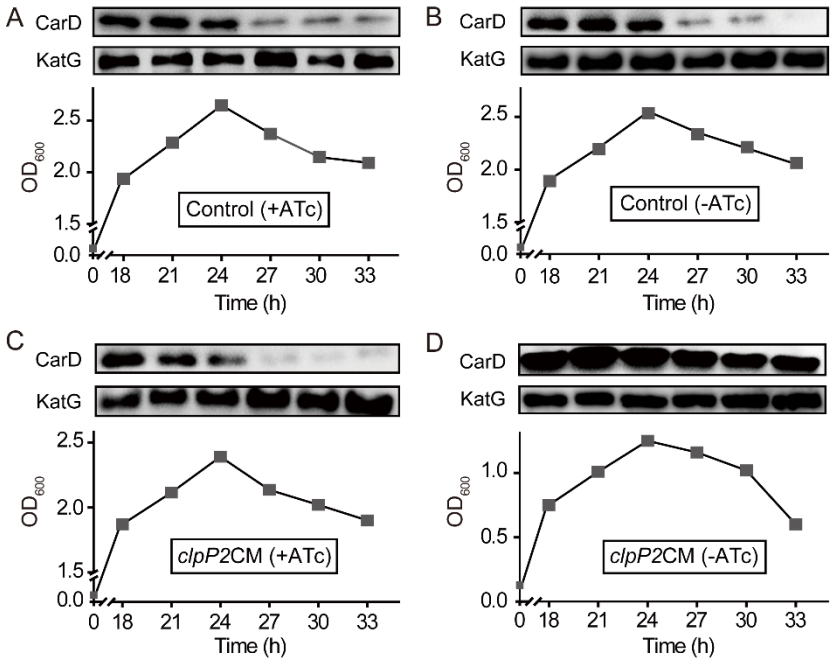

Fig. 3. Clp protease is responsible for CarD degradation in stationary phase. A and **B,** the intracellular CarD levels at different time points of the ATc-induced and ATc-uninduced control strain cells (Ms/pRH2502-*clpP2*), respectively. **C** and **D,** the CarD levels at different time points of the ATc-induced and ATc-uninduced *clpP2*CM (*clpP2* conditional mutant) cells, respectively. KatG was used as the control in the Western blot experiments.

To investigate whether Clp protease is also required for degrading intracellular CarD under starvation conditions, we carried out a series of starvation experiments on *clpP2*CM cells harvested from the MEP. The results showed that CarD was rapidly degraded when the ATc-induced *clpP*2CM cells were starved in PBS for 4 h, while in the ATc-uninduced *clpP*2CM cells CarD was not degraded (Fig. S4A). To further confirm this result, we carried out *in vitro* CarD degradation experiments by incubating the purified CarD protein with the total protein extracts of ATc-induced and ATc-uninduced *clpP*2CM cells, respectively, and measured the extent of CarD degradation. The results showed that the untreated total proteins extracted from ATc-induced *clpP*2CM cells could degrade the purified CarD (Fig. S4B), while the protein extracted from ATc-uninduced *clpP*2CM cells could not (Fig. S4C). This result was consistent with the experimental data described above, allowing us to conclude that Clp protease was responsible for the degradation of CarD under starvation conditions.

Starvation induces the transcription of antisense RNA of *carD*

Next, we wanted to know whether the intracellular CarD content is subject to other types of regulation other than degradation by Clp protease. After mining our previously published RNA-seq data of strain mc^2155 (27), we identified an antisense RNA transcribed from the complementary strand of the *carD-ispD* operon. As shown in Fig. 4A, this antisense RNA (named AscarD) is partially complementary to the coding region of *ispD* but fully complementary to the coding region of *carD*. The RNA-seq data also showed that AscarD was specifically induced in the MSP (Fig. 4A). To confirm this phenomenon, we examined the transcriptional level of AscarD at different growth phases by RT-PCR, and the results showed that AscarD level during the MSP was significantly higher than that in the MEP (Fig. S5), which is consistent with the RNA-seq data.

To better characterize AscarD, we determined its transcriptional start site (TSS) by carrying out the 5'-RACE (5'-Rapid Amplification of cDNA Ends) experiment (Fig. 4B). The TSS identified by 5'-RACE was consistent with that revealed by the RNA-seq data. We also discovered potential SigF-recognized -10 and -35 motifs upstream of the identified TSS (Fig. 4C). SigF is an alternative sigma factor that is active in the stationary phase, which is consistent with the transcriptional pattern of AscarD, suggesting that the transcription of *ascarD* is controlled by SigF. To verify this hypothesis, we examined the transcriptional level of *ascarD* in a *sigF* mutant (Δ*sigF*). As shown in Fig. 4D, only a very low AscarD transcriptional level could be detected in the Δ*sigF* strain in the MEP, and transition to the MSP could not induce it either. These data indicate that the expression of *ascarD* is regulated by SigF.

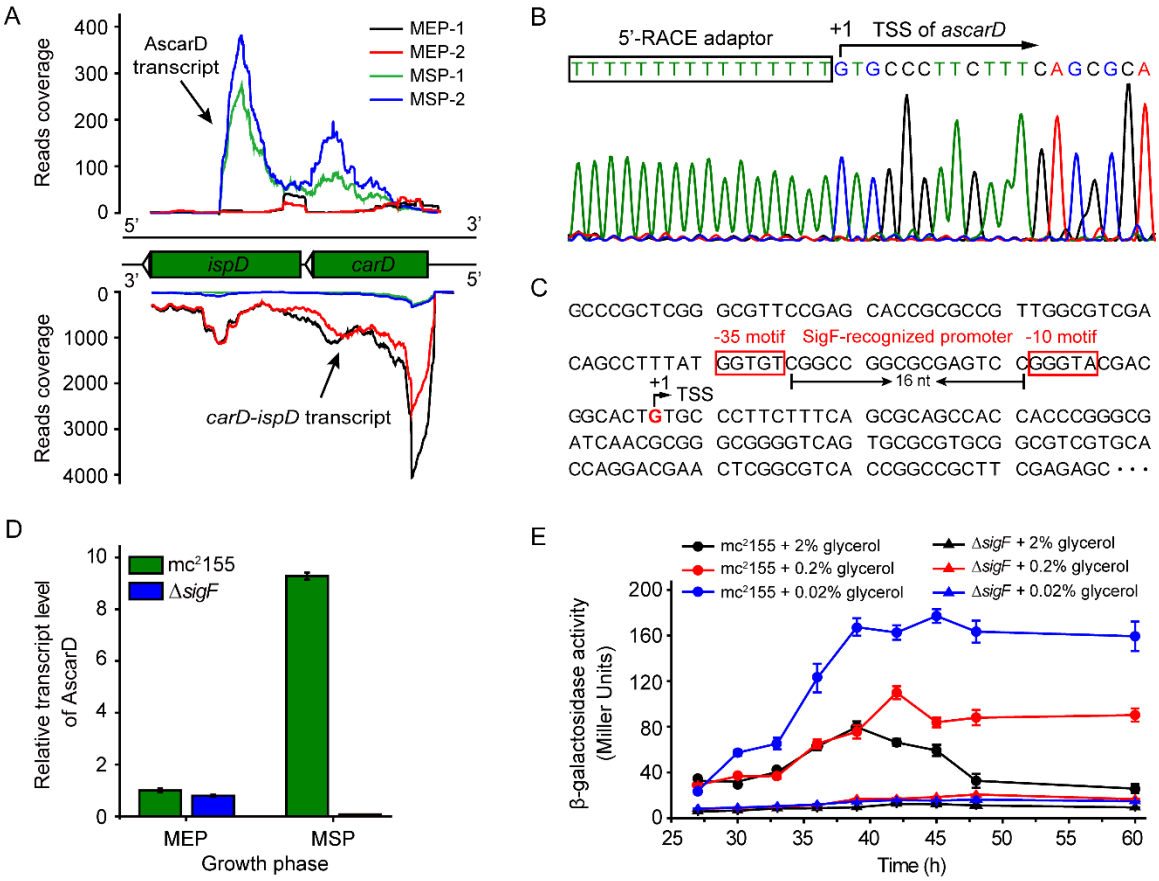

Fig. 4. Identification and characterization of AscarD. A, transcriptional landscapes of *carD-ispD* transcript and AscarD. Red and black lines represent exponential-phase cells, blue and green lines are from stationary-phase cells. Extensions of -1 and -2 represent two biological replicates. **B,** mapping of the transcriptional start site (TSS) of AscarD. The lower four-color chromatogram shows the results of Sanger sequencing, the corresponding DNA sequence is displayed on the upper layer. The 5'-RACE adaptor sequence is framed by a black rectangle, and TSS is indicated by a black arrow. **C,** potential SigF-recognized -10 and -35 motifs (GGTGT-N_{16}-GGGTA) upstream of the identified TSS are indicated with red rectangles. **D,** transcriptional levels of AscarD in mc^2155 and Δ*sigF* strains as measured by qRT-PCR. **E,** promoter activities of *ascarD* in mc^2155 and Δ*sigF* strains carrying a β-galactosidase-encoding reporter plasmid. Error bars indicate the standard deviation of three biological replicates.

Further, since *ascarD* was highly expressed during the stationary phase, we speculated that transcription of *ascarD* could be also subject to carbon starvation. To verify this idea,

we carried out *lacZ* reporter assays to examine the *ascarD* promoter activity under different carbon source (glycerol) concentrations. As shown in Fig. 4E, the *ascarD* promoter activity gradually increased as the glycerol concentrations decreased. This indicates that *ascarD* could indeed be induced under carbon starvation conditions; however, in the Δ*sigF* strain, the expression of *ascarD* did not respond to the glycerol concentration (Fig. 4E). This indicates that the response of *ascarD* to low carbon requires the presence of SigF, which is consistent with the above results. Thus, we confirm that the transcription of *ascarD* was highly induced in response to starvation in a SigF-dependent manner.

AscarD inhibits biosynthesis of CarD protein

Expression of AscarD was highly induced in response to starvation, while the CarD protein level was sharply reduced, suggesting that AscarD could be involved in regulating *carD* expression. To clarify this issue, we constructed the control plasmid P_{PU} as well as the target plasmid P_{PUC} (Methods), and carried out a series of *lacZ* reporter assays (Fig. 5A). AscarD was expected to target the transcript obtained from the P_{PUC} plasmid but not the transcript from the P_{PU} plasmid. We then transformed the two plasmids into the *ascarD* overexpression strain (*ascarD*$_{OE}$), *ascarD* knockdown strain (*ascarD*$_{KD}$), and the control strain (mc^2155 with pMV261 empty vector), respectively, and examined their β-galactosidase activities. The β-galactosidase activities among the three strains transferred with the control plasmid P_{PU} did not differ much (Fig. 5B). However, for those transferred with target plasmid P_{PUC}, the β-galactosidase activity was significantly reduced in the *ascarD*$_{OE}$ strain, while significantly increased in the *ascarD*$_{KD}$ strain compared to the control strain (Fig. 5B). These results indicate that AscarD inhibited the expression of the *carD-lacZ* transcript by targeting the *carD* region.

Additionally, we modified the P_{PUC} plasmid by fusing the original sequence (P_{ascarD}) and the mutated sequence (P_{ascarD}M) of the *ascarD* promoter region upstream of *lacZ* to construct the P_{PUCP} and P_{PUCP}* plasmids, respectively (Fig. 5C). This type of construct can reflect the native interaction between the AscarD and *carD* transcripts. As shown in Fig. 5D, mycobacterial cells transformed with the P_{PUCP}* plasmid exhibited higher β-galactosidase activity than those with the P_{PUCP} plasmid. This result indicates that the

mutated *ascarD* promoter failed to initiate the AscarD transcription and therefore could not suppress the expression of the *carD-lacZ* transcript. Based on the above *lacZ* reporter assay data, we conclude that AscarD negatively regulates the expression of *carD*.

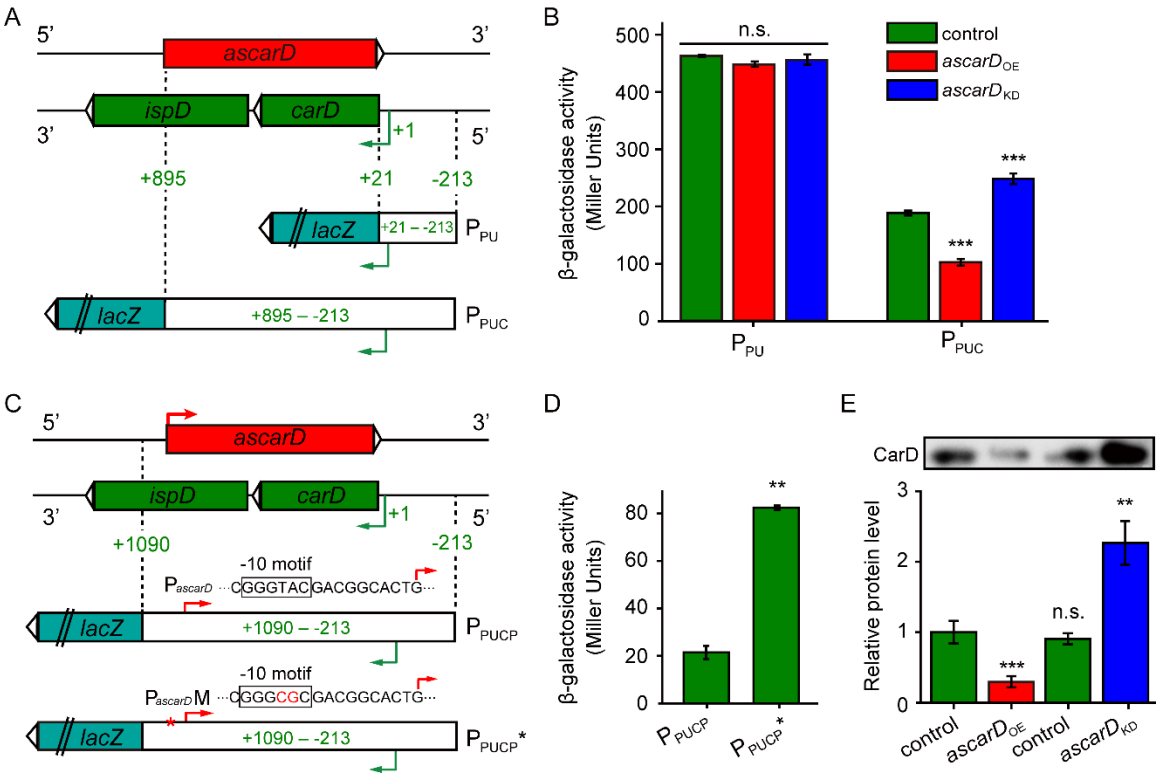

Fig. 5. AscarD negatively regulates *carD*. A and **C,** a schematic diagram of P_{PU}, P_{PUC}, P_{PUCP}, and P_{PUCP}* plasmid construction (see a detailed description in Methods). **B,** β-galactosidase activities of different strains transformed with P_{PU} or P_{PUC} plasmid. **D,** β-galactosidase activities of mc^2155 strain transformed with P_{PUCP} and P_{PUCP}* plasmid. **E,** CarD protein levels in different strains. Mycobacterial cells were harvested at the MSP. The upper part shows the Western blot with CarD levels, the histogram below it shows the quantitative statistics of Western blot results (Error bars indicate standard deviation of three biological replicates).

Further, we examined the effect of AscarD content on the CarD protein levels in strains with different AscarD levels. As shown in Fig. 5E, compared to the control strain, the CarD level in the *ascarD*$_{OE}$ strain was reduced, while in the *ascarD*$_{KD}$ strain it was significantly increased. This result indicates that AscarD inhibits the synthesis of CarD, which is consistent with the *lacZ* reporter assay data described above. It is important to point out

that the inhibitory effect of antisense RNA on target genes can occur at the post-transcriptional level (reducing transcript stability) and/or the translational level (inhibiting the transcript translation) (28). To determine the inhibition mode, we measured the transcript level of *carD* in the *ascarD*$_{OE}$ strain. The *carD* transcript levels were significantly higher in the *ascarD*$_{OE}$ strain than those in the control strain (Fig. S6), illustrating that overexpressed AscarD increases, rather than decreases, the stability of *carD* transcripts. Since AscarD only reduces the CarD protein level, but not the transcript level, we believe that AscarD inhibits *carD* expression at the translational level.

Discussion

In this paper, we present an in-depth study on the regulation of CarD expression and demonstrate that CarD is synergistically regulated by both AscarD antisense RNA and Clp protease under starvation conditions. Based on these results, along with those published by others, we propose a new mechanism for mycobacterial adaption to the starvation conditions, namely, that mycobacterial cells adjust their transcriptional and translational rates by regulating the CarD levels in response to the environmental conditions (Fig. 6). Under abundant nutrition, mycobacterial cells use CarD to stabilize RPo (7-9, 29), promoting the transcription of rRNA and other related genes (9, 16, 18) to ensure vigorous cell growth (Fig. 6A). However, when external nutrition gets scarce, SigF-regulated expression of AscarD is induced and it hybridizes with *carD* mRNA to prevent the translation of CarD protein (Fig. 4 and 5). Meanwhile, the residual intracellular CarD protein is rapidly degraded by Clp protease to keep CarD at a very low level (Fig. 1, 2, 3), which potentially reduces the stability of RPo and diminishes the synthesis of rRNA; these processes combine to slow down the rate of transcription and translation of mycobacterial cells (Fig. 6B). When nutrients are available, AscarD transcription is inhibited and *carD* mRNA gets translated to resume the normal CarD level and ensure the re-growth of mycobacterial cells (Fig. 1F). Overall, these findings contribute to a better understanding of the mechanisms of mycobacterial adaptation to starvation and provide certain clues that might help in the treatment of tuberculosis.

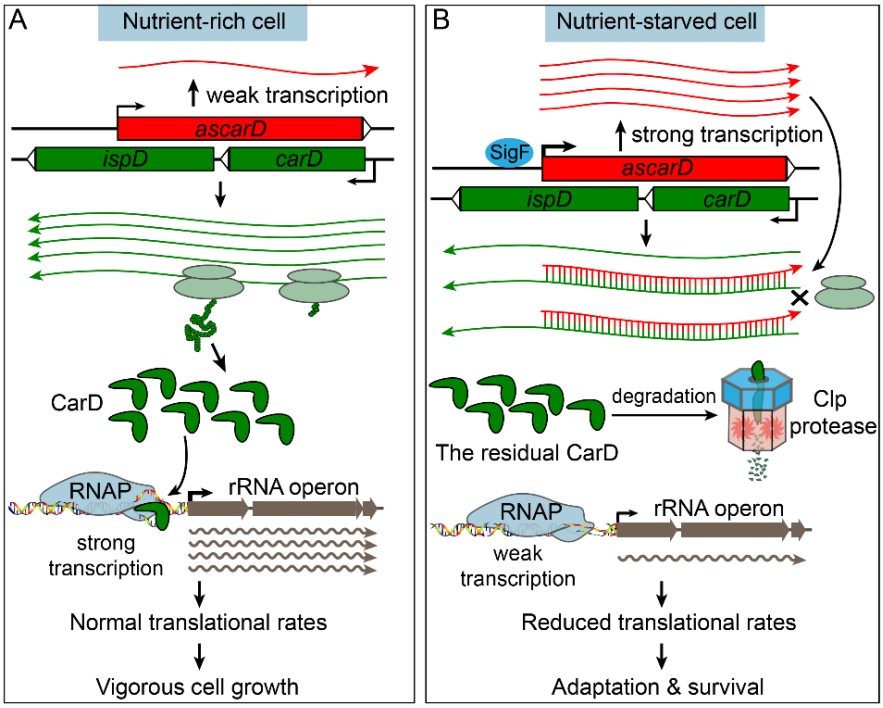

Fig. 6. AscarD and Clp protease work synergistically to regulate CarD-mediated starvation response. A and **B** represent the nutrient-rich and nutrient-starved cells, respectively.

Mycobacterial CarD defines a distinct adaptive response mechanism

Before this work, the best-known starvation response mechanism in *Mycobacterium* was the stringent response mediated by (p)ppGpp. Yet, its detailed mechanism is still not entirely clear today, although it is considered similar to the well-characterized stringent response mechanisms reported in *E. coli* and *B. subtilis* (30-32). In *E. coli*, (p)ppGpp is synthesized in large quantities in response to starvation and directly interacts with RNAP to destabilize the RPo formed on rRNA genes and consequently reduces the rRNA synthesis (4). However, (p)ppGpp in *B. subtilis* does not directly interact with RNAP but instead decreases the intracellular GTP content to destabilize the RPo formed on the genes that start from guanine, such as the rRNA genes (33, 34). In mycobacteria, the exact effect and role of ppGpp on rRNA transcription is unclear, but (p)ppGpp likely inhibits the transcription of mycobacterial rRNA by affecting the stability of RPo (32), which is similar to the CarD-mediated starvation response. Of course, these two mechanisms also have their unique features. First, (p)ppGpp reduces the stability of RPo (30, 35), while CarD enhances

its stability (7-9, 29); second, (p)ppGpp is rapidly synthesized in response to starvation, while CarD is quickly degraded under starvation conditions. Despite the differences between the two mechanisms, they basically work in the same way and ultimately help the mycobacterial cells adapt to starvation by reducing the rRNA synthesis. It should be noted that Stallings and colleagues previously reported that CarD is required for stringent response in *M. smegmatis* (14). However, our data showed that CarD is rapidly degraded under starvation conditions where stringent response usually occurs. This may seem to contradict the previous study, but since both CarD and (p)ppGpp interact with RNAP (4, 14), the two molecules might have complex effects on RNAP that remain to be disentangled.

In addition, our study found that mycobacterial cells reduce CarD levels in response to hypoxic condition. By analyzing the RNA-seq data for *M. tuberculosis* (12), we found that 18 of the top 20 up-regulated genes (Table S1) in $CarD^{K125A}$ strain (a mutant with predicted weakened affinity of CarD to DNA) belong to the previously identified dormancy regulon (36), and 31 out of 48 dormancy regulon genes are significantly up-regulated. These data indicate that CarD represses the expression of dormancy regulon genes, and the reduction of CarD level during starvation or hypoxia may derepress these genes and facilitate mycobacterial dormancy. Since pathogenic mycobacteria usually live in a nutrient-deprived and hypoxic environment after infecting the host, we believe that CarD plays an important role in the dormancy and persistence of pathogenic mycobacteria in the host cells. Taken together, the CarD-mediated mycobacterial adaptive response mechanism is multi-faceted; reduction of CarD not only down-regulates the transcription of rRNA to help mycobacterial cells adapt to nutritional starvation, but also enhances the expression of dormancy regulon genes to help pathogenic mycobacteria entering into a dormant state.

The mechanism of specific degradation of CarD under starvation condition

The rapid decrease of CarD protein levels under starvation was caused by its degradation by Clp protease. But after analyzing the transcriptomics (27) and proteomics (37) data, we found that Clp protease-related genes are expressed normally in the exponential phase. Therefore, we speculate that the intracellular CarD in the exponential phase may be protected by a certain complex, which is no longer in force after the mycobacterial cells

entered the stationary phase. It is worth noting that Garner et al. previous research suggested that CarD-RNAP interaction protects CarD from proteolytic degradation in *M. tuberculosis* (15). Therefore, we speculate that it maybe RNAP that protect CarD in the exponential phase. After entering the stationary phase, CarD may be detached from RNAP through unknown mechanisms and be rapidly degraded by Clp protease. Of course, there may be other reasons why CarD in the exponential phase was not degraded. First, besides Clp protease, degradation of CarD may require an adaptor protein. For example, in *Caulobacter crescentus*, CpdR directly controls PdeA degradation by acting as a phosphorylation-dependent adaptor protein for the ClpXP protease (38). We speculate that there is possibly an adaptor protein that recognizes CarD under the starvation condition and delivers it to the Clp protease for degradation. Second, Clp protease may only degrade the modified (or unmodified) CarD. For example, certain protein substrates in *B. subtilis* are degraded by Clp protease only after their arginine residues are phosphorylated (39). CarD might undergo a similar structural modification (or un-modification) under the starvation condition, which is specifically recognized and degraded by Clp protease.

The regulation of *carD* by AscarD

The regulatory mechanism of AscarD is somewhat similar to that of *E. coli* antisense RNA GadY, which is also induced during stationary phase and enhances the stability of the target mRNA (40). GadY binding to the intergenic region of the bicistronic transcript *gadX-gadW* induces cleavage of this transcript by endoribonucleases, mainly RNase III, resulting in two stable transcripts *gadX* and *gadW* (41, 42). AscarD likely works similarly to GadY since the base paring of AscarD to the *carD-ispD* transcript also covers its intergenic region. Moreover, mycobacteria possess a variety of endoribonucleases, including RNase III, which may direct *carD-ispD* processing. However, AscarD and GadY do differ in some respects. GadY is short (105 nt processed to 90- and 59-nt forms) and only complementary to the intergenic region of *gadX-gadW*, while AscarD is relatively long (~800 nt) and complementary to the *carD/ispD* coding region. Besides, GadY increases the protein yield of GadX, while AscarD reduces the protein yield of CarD. We speculate that the inhibitory effect of AscarD on CarD protein synthesis may be due to the

complete base pairing between AscarD and *carD* mRNA, which probably causes a block in ribosome binding. Future studies should clarify the exact mechanism by how AscarD affects CarD's stability and translation and help us better understand the biological significance of AscarD.

The effects of CarD regulation on RbpA expression

In contrast to CarD, the protein levels of RbpA, another RNAP accessory transcription factor in mycobacteria, increase during the stationary phase (43). This helps the mycobacterial cells to maintain the (slow) growth in the stationary phase, as they still need a certain number of functioning ribosomes and a certain (low) level of rRNA synthesis to survive. RbpA exhibits a specific interaction with σ^B (44) to potently regulate σ^B-dependent genes. σ^B is induced in the stationary phase (45) and participates substantially in the stationary-phase survival and stress response of mycobacterial cells (46, 47). Therefore, we believe that the increase of RbpA amount in the stationary phase is beneficial as it elevates the expression of σ^B-dependent stress response genes and helps mycobacterial cells better adapt to adverse environments.

Materials and Methods

Bacterial strains and growth conditions

E. coli strains were cultivated in lysogeny broth (LB) medium at 37°C. *M. smegmatis* mc^2155 wild-type strain (48) and its derivatives were grown at 37°C in Middlebrook 7H9 medium supplemented with 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80, or on Middlebrook 7H10 agar supplemented with 0.5% (v/v) glycerol. *M. bovis* BCG and *M. tuberculosis* H37Ra strains (49) were grown at 37°C in 7H9 medium supplemented with 0.5% glycerol, 0.05% Tween 80 and 10% OADC (oleic acid, albumin, dextrose, and catalase), or on Middlebrook 7H11 agar supplemented with 0.5% glycerol and 10% OADC. When required, antibiotics were added at the following concentrations: kanamycin (Kan), 25 μg/mL; hygromycin (Hyg), 50 μg/mL; streptomycin (Str), 10 μg/mL.

Hartmans-de Bont (HDB) minimal medium, prepared according to reference (22), was used for starvation experiments. Briefly, 1 L of HDB medium contained: 10 mg of EDTA, 100 mg of $MgCl_2 \cdot 6H_2O$, 1 mg of $CaCl_2 \cdot 2H_2O$, 0.2 mg of $NaMoO_4 \cdot 2H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, 1 mg of $MnCl_2 \cdot 2H_2O$, 2 mg of $ZnSO_4 \cdot 7H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 1.55 g of K_2HPO_4, 0.85 g of NaH_2PO_4, 2.0 g of $(NH_4)_2SO_4$, 0.2% glycerol (v/v), and 0.05% Tween 80 (v/v). For the carbon starvation experiment, glycerol was removed; for the nitrogen starvation experiment, $(NH_4)_2SO_4$ was removed; for the phosphorus starvation experiment, both K_2HPO_4 and NaH_2PO_4 were removed, while 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS) was added to replace lost buffering capacity.

Stimulation and starvation experiments

Mycobacterial cells were first grown to mid-exponential phase (MEP) in the normal 7H9 medium. For genotoxic reagent stimulation experiments, 10 mM H_2O_2, 0.1% methyl methanesulphonate (MMS), and 10 mg/mL of ciprofloxacin (CIP) were separately added to the MEP mc^2155 culture, and maintained in roller bottle culture for additional 4 h. For the PBS starvation experiment, the MEP mc^2155 cells were harvested, resuspended in PBS supplemented with 0.05% Tween 80, and maintained in roller bottle culture for 4 h. For the carbon-, nitrogen-, and phosphorus-starvation experiments, harvested MEP cells were resuspended in the HDB medium with carbon, nitrogen, or phosphorus removed, respectively, and maintained in roller bottle culture for 4 h for the mc^2155 cells, or 24 h for the BCG and H37Ra cells. For the nutrient supplemented experiments, the above-mentioned starved cultures were supplemented with the corresponding nutrients and maintained in roller bottle culture for additional 4 h for the mc^2155 cells, or 24 h for the BCG and H37Ra cells. For the acid stimulation experiments, the harvested MEP cells were resuspended in the HDB medium with low pH value (pH 4.5) and maintained in roller bottle culture for 4 h for the mc^2155 cells, or 24 h for the BCG and H37Ra cells.

For anaerobic experiments, the modified Wayne model (50) were used. Briefly, 150 mL standard serum bottles containing 100 mL of 7H9 medium were used, in which methylene blue was added to the final concentration of 2 μg/mL to indicate oxygen content. The

harvested MEP cells were re-inoculated into the above serum bottles to make the final OD_{600} of 0.02. Then, the serum bottles were sealed with butyl rubber stoppers, closed tightly with screw-caps, and incubated at 37°C with shaking. The mc^2155 cells were harvested 10 h after the blue color disappeared completely, and the BCG and H37Ra cells were harvested 48 h after the blue color completely disappeared. For the re-aeration experiments, the above-mentioned anaerobic cultures were transferred to roller bottles and harvested after the mycobacterial cells regrow.

RNA isolation, reverse transcription and qRT-PCR

Total RNA was extracted by TRIzol method as described previously (27). The quality and concentration of total RNA were analyzed by NanoDrop 2000 (Thermo Scientific, USA). Reverse transcription experiment was conducted with the commercially available PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology, Japan) according to the manufacturer's instructions. The cDNAs of *carD* and *ascarD* were synthesized using gene-specific primers RT-*carD*-R or RT-*ascarD*-R, respectively (Table S3), which can distinguish the two transcripts. For qRT-PCR, *sigA* was used as the internal reference gene and the experiment was carried out according to reference (51).

CRISPRi-mediated gene knockdown strategy

CRISPR/dCas9-mediated gene knockdown strategy (52) was carried out to construct the $AscarD_{KD}$ strain. Briefly, pRH2502 plasmid containing *int* and *dcas9* genes was integrated into the mc^2155 genome to generate Ms/pRH2502 strain (Table S2). pRH2521 plasmid containing the small guide RNA (sgRNA) targeting *ascarD* was transferred into Ms/pRH2502 strain to obtain the final $AscarD_{KD}$ strain (Table S2). The expression of both *dcas9* and sgRNA requires the induction by anhydrotetracycline (ATc). The transcription of the target gene (*ascarD*) was inhibited with the induction by 50 ng/mL of ATc, and the inhibition efficiency was assessed by qRT-PCR. It is worth noting that dCas9:sgRNA complex exhibits a strong inhibitory effect on the expression of a gene after it combines with the coding strand of the gene, but almost does not affect the expression of the gene when it combines with the template strand. The sgRNA we designed is combined with the coding strand of *ascarD* (that is, the template strand of *ispD*), so it has a strong inhibitory

effect on the transcription of *ascarD* (reduced 18.8 ± 2.6 times as quantitated by qRT-PCR), but has almost no effect on the transcription of *ispD*. The inhibition efficiency is shown in Fig. S7, and all related primers are listed in Table S3.

CRISPR/Cpf1-mediated mutagenesis

CRISPR/Cpf1-mediated mutagenesis was carried out as described previously (26). This system consists of three parts: Cpf1, crRNA, and ssDNA oligonucleotide. The pJV53-*cpf1* plasmid was employed for the inducible (with 50 ng/mL ATc) expression of Cpf1. The pCR-Hyg-crRNA plasmid was used for the constitutive expression of crRNA. For the ssDNA oligonucleotide, a 59-nt oligonucleotide spanning the genome broken site and targeting the lagging strand of DNA replication was synthesized. The expected mutation was introduced into the middle of the oligonucleotide sequences. For gene mutation, pJV53-Cpf1 was first transformed into mc^2155 to generate the recombinant strain Ms/pJV53-Cpf1. The recombinant vector pCR-Hyg-crRNA and the 59-nt oligonucleotide were then co-transformed into Ms/pJV53-Cpf1 strain. Mycobacterial cells were then spread onto 7H10 plates supplemented with the corresponding antibiotics and set to 37°C for growth. Three days later, clones were picked and verified by PCR and sequencing. The primers and DNA oligonucleotides used are listed in Table S3.

Construction of the *clpP*2 conditional mutant

Unlike *E. coli* ClpP that comprises a homo-tetradecamer, mycobacterial ClpP forms a hetero-tetradecamer comprising two proteases, ClpP1 and ClpP2, which are both essential, and deletion of either one of them leads to the loss of degradative activity (25). Therefore, for *clpP2* conditional mutant (*clpP*2CM) construction (Fig. S3), an exogenous *clpP*2 gene amplified with *clpP2*-F/R primer pair (Table S3) was ligated to the pRH2502 integration plasmid to obtain pRH2502-*clpP2* recombinant plasmid, in which *clpP2* is under the control of ATc-inducible promoter $P_{UV15tetO}$. The pRH2502-*clpP2* plasmid was then transformed and integrated into the mc^2155 genome by *attB*-*attP* mediated site-specific recombination, to obtain Ms/pRH2502-*clpP2* strain (used as the control strain in Fig. 3A and 3B). Finally, the endogenous *clpP2* gene on Ms/pRH2502-*clpP2* genome was mutated (pre-translational termination) using the CRISPR/Cpf1-mediated mutagenesis. Thus,

*clpP*2 could be expressed normally in the *clpP*2CM strain only upon addition of 50 ng/mL ATc, but could not do so when ATc was absent. It should be noted that, in principle, the *clpP2*CM strain cannot grow in the 7H9 medium without ATc. But when we first cultivated the *clpP2*CM cells to exponential phase in ATc-containing 7H9 medium, then harvested the cells, washed them, and inoculated them into ATc-free 7H9 medium (initial OD_{600} = 0.1), *clpP2*CM cells can grow slowly (Fig. 3D).

β-galactosidase experiment

For P_{PU} construction, the -213 – +21 region of *carD*, containing the *carD* promoter and 5'-untranslated region (5'-UTR), was fused to *lacZ*; for P_{PUC}, the -213 – +895 region of *carD*, containing the *carD* promoter, 5'-UTR, and the *carD* coding sequence (CDS), was fused to *lacZ*; for P_{PUCP}, the -213 – +1090 region of *carD*, containing the *carD* promoter, 5'-UTR, *carD* CDS, and *ascarD* promoter on the antisense strand, was fused to *lacZ*; for P_{PUCP}*, the modified -213 – +1090 region of *carD*, containing the *carD* promoter, 5'-UTR, *carD* CDS, and the mutated *ascarD* promoter (P_{ascarD}M) on the antisense strand, was fused to *lacZ*. To measure the β-galactosidase activity, mc^2155 derivatives were grown at 37°C. Mycobacterial cells equivalent of OD_{600}=1 (e.g., 2 mL of each culture with OD_{600} = 0.5) were harvested at the given times followed by washing with PBS. The detailed steps for β-galactosidase activity determination were carried out according to references (51, 53).

5'-Rapid amplification of cDNA ends (5'-RACE)

To identify the TSS of *ascarD*, 5'-RACE analysis was performed with RNA extracted from mc^2155 cells at mid-stationary phase grown in 7H9 medium. The 5'-RACE experiment was performed as described previously (54). The primers used are listed in Table S3.

Western blot

For the Western blot, CarD was detected using CarD-specific rabbit polyclonal antibody that were prepared by Dia-An Biotech, Inc. (Wuhan, China). The specificity of the CarD polyclonal antibody is shown in Fig. S8. For SigA detection, His×6 tag was fused to the C-terminus of SigA by inserting its coding sequence immediately upstream of the *sigA* stop codon in the mc^2155 genome, and the modified SigA-His×6 protein was detected using

rabbit polyclonal antibody to His×6 (Yeasen Biotech Co., Shanghai, China). Generally, KatG was used as the internal reference protein in Western blot experiments. However, since its level is affected by oxidative stress, SigA was used as an internal reference protein in the genotoxic stress stimulation experiment (Fig. 1B). The detailed procedures of Western blot were as described previously (55).

CarD purification and *in vitro* protease degradation experiment

The CarD protein used in the *in vitro* degradation assay was purified from *M. smegmatis*. *carD* coding sequence fused with a His×6 tag sequence was cloned into pMV261 plasmid and transformed into the mc^2155 to obtain $carD_{OE}$ (Table S2). CarD-His protein was purified by affinity Ni^{2+}-NTA column. The purified CarD protein was used as a substrate for *in vitro* protease degradation experiments. Mycobacterial cells harvested from the stationary phase were subjected to ultrasonication to obtain the total protein lysate, which was used to degrade the purified CarD protein. For *in vitro* degradation assay, 2 μg of CarD protein were incubated with 16 μg of either the total protein extract, boiled total protein extract (boiling for 15 min), or the protein extract containing 20 mM of EDTA, respectively. Samples were incubated at room temperature for 10 h, followed by Western blot analysis.

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Author Contributions

X.F.L., F.C and J.H. designed the study. F.C., J.F.X., X.Y.L., X.F.L., and B.T.A. performed the experiments. F.C., X.F.L., X.C., and Q.T. analyzed the data. X.F.L., F.C., S.H.C. and M.Y.G. wrote the original draft., M.Y.G., S.H.C., and J.H. prepared the final manuscript.

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