

mRNA Degradation Rates Are Coupled to Metabolic Status in *Mycobacterium*
smegmatis

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Running Head: mRNA Stability is Coupled to Metabolic Status

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ABSTRACT

The success of *Mycobacterium tuberculosis* (Mtb) as a human pathogen is due in part to its ability to survive stress conditions, such as hypoxia or nutrient deprivation, by entering non-growing states. In these low-metabolic states, Mtb can tolerate antibiotics and develop genetically encoded antibiotic resistance, making its metabolic adaptation to stress crucial for survival. Numerous bacteria, including Mtb, have been shown to reduce their rates of mRNA degradation under growth limitation and stress. While the existence of this response appears to be conserved across species, the underlying bacterial mRNA stabilization mechanisms remain unknown. To better understand the biology of non-growing mycobacteria, we sought to identify the mechanistic basis of mRNA stabilization in the non-pathogenic model *Mycobacterium smegmatis*. We found that mRNA half-life was responsive to energy stress, with carbon starvation and hypoxia causing global mRNA stabilization. This global stabilization was rapidly reversed when hypoxia-adapted cultures were re-exposed to oxygen, even in the absence of new transcription. The stringent response and RNase levels did not explain mRNA stabilization, nor did transcript abundance. This led us to hypothesize that metabolic changes during growth cessation impact the activity of degradation proteins, increasing mRNA stability. Indeed, bedaquiline and isoniazid, two drugs with opposing effects on cellular energy status, had opposite effects on mRNA half-lives in growth-arrested cells. Taken together, our results indicate that mRNA stability in mycobacteria is not directly regulated by growth status, but rather is dependent on the status of energy metabolism.

IMPORTANCE

The logistics of tuberculosis therapy are difficult, requiring multiple drugs for many months. Mtb survives in part by entering non-growing states in which it is metabolically less active, and thus

less susceptible to antibiotics. Basic knowledge on how Mtb survives during these low-metabolic states is incomplete, and we hypothesize that optimized energy resource management is important. Here we report that slowed mRNA turnover is a common feature of mycobacteria under energy stress, but is not dependent on the mechanisms that have generally been postulated in the literature. Finally, we found that mRNA stability and growth status can be decoupled by a drug that causes growth arrest but increases metabolic activity, indicating that mRNA stability responds to metabolic status rather than to growth rate per se. Our findings suggest a need to re-orient studies of global mRNA stabilization to identify novel mechanisms that are presumably responsible.

INTRODUCTION

Most bacteria periodically face environments that are unfavorable for growth. To overcome such challenges, bacteria must tune their gene expression and energy usage. Regulation of mRNA turnover can contribute to both of these. However, the mechanisms by which mRNA turnover is carried out and regulated remain poorly understood, particularly in mycobacteria.

During infection, the human pathogen *Mycobacterium tuberculosis* (Mtb) faces not only the immune response and antibiotics, but also non-optimal microenvironments such as hypoxia and starvation (1, 2). Regulation of mRNA turnover appears to contribute to adaptation to such conditions. A global study of mRNA decay in Mtb showed a dramatic increase in transcriptome stability (increased mRNA half-lives) in response to hypoxia, compared to aerobic growth (3). This suggests that mRNA stabilization contributes to energy conservation in the energy-limited environments that Mtb encounters during infection. Similar responses have been shown for other

bacteria under conditions that slow or halt growth, including carbon deprivation, stationary phase, and temperature shock (4-13). However, the mechanisms responsible for global regulation of mRNA stability in prokaryotes remain unknown.

A conventional model for RNA decay in *E. coli* starts with endonucleolytic cleavage by RNase E, particularly in 5' monophosphorylated mRNAs (14-16). The resulting fragments are further cleaved by RNase E, producing fragments that are fully degraded by exonucleases such as PNPase, RNase II, and RNase R (17, 18). mRNA degradation is coordinated by formation of a complex known as the degradosome. In *E. coli*, RNase E serves as the scaffold for degradosomes containing RNA helicases, the glycolytic enzyme enolase, and PNPase (19-23). Other organisms that encode RNase E form similar degradosomes (24, 25). In bacteria lacking RNase E, other endonucleases assume the scaffold function (26-28). Mycobacteria encode RNase E, but efforts to define the mycobacterial degradosome have produced inconsistent results (29, 30). It is unclear if degradosome reorganization or dissolution contribute to the global regulation of mRNA degradation in any bacteria. Interestingly, the importance of degradosome formation in *E. coli* varies depending on the carbon sources provided, suggesting links between RNA degradation and metabolic capabilities (31). Furthermore, the chaperones DnaK and CsdA associate with degradosomes in *E. coli* under certain stresses (20, 32, 33).

Global transcript stabilization in stressed bacteria could plausibly result from reduced RNase abundance, reduced RNase activity, and/or reduced accessibility of transcripts to degradation proteins. In *E. coli* multiple stressors upregulate RNase R, possibly to mitigate ribosome misassembly (34, 35), and RNase III levels decrease under cold-shock and stationary phase (36). Surprisingly, protein levels for most putative RNA degradation proteins in Mtb remain unaltered under hypoxic conditions (37), suggesting that mRNA degradation is not necessarily regulated at

the level of RNase abundance in mycobacteria. However, there is evidence that RNase activity may be regulated. For example, proteins such as RraA and RraB can alter the function of the RNase E-based degradosome in *E. coli* (38). Translating ribosomes can mask mRNA cleavage sites and stabilize mRNAs (39). In *Caulobacter crescentus*, subcellular localization of mRNA degradation proteins may affect global mRNA stability (40, 41). Furthermore, in some actinomycetes, PNPase might be regulated by the stringent response alarmones guanosine-3'-diphosphate-5'-triphosphate (pppGpp) and/or guanosine-3',5' -bisphosphate (ppGpp), collectively referred to as (p)ppGpp (42, 43). Many bacteria synthesize (p)ppGpp in response to energy stress (44-47), where it generally facilitates adaptation by upregulating stress-associated genes and downregulating those associated with growth (46, 48-52). (p)ppGpp was reported to inhibit the activity of PNPase in two actinomycetes, *Streptomyces coelicolor* and *Nonomuraea* (42, 43), suggesting that the stringent response could directly stabilize mRNA as part of a broader response to energy starvation.

Another explanation for stress-induced transcript stabilization could be that reduced transcript abundance directly leads to increased transcript stability. mRNA abundance and half-life were reported to be inversely correlated in multiple bacteria including *Mtb* (3, 8, 53, 54), and mRNA abundance is lower on a per-cell basis for most transcripts in non-growing bacteria. Nevertheless, the causal relationships between translation, mRNA abundance, RNase expression, and mRNA stability in non-growing bacteria remain largely untested.

Given the importance of adaptation to energy starvation for mycobacteria, we sought to investigate the mechanisms by which mRNA stability is globally regulated. Here we show that the global mRNA stabilization response occurs also in *Mycobacterium smegmatis*—a non-pathogenic model commonly used to study the basic biology of mycobacteria —under hypoxia

and carbon starvation. Remarkably, we found that hypoxia-induced mRNA stability is rapidly reversible, with re-aeration causing immediate mRNA destabilization even in the absence of protein synthesis. As expected, transcript levels from hypoxic cells were lower on a per-cell basis compared to those from aerated cultures. However, our data are inconsistent with a model in which mRNA abundance dictates degradation rate as has been shown for log-phase *E. coli* (53) and *Lactococcus lactis* (54). Instead, our findings support the idea that mRNA stability is rapidly tuned in response to alterations in energy metabolism. This effect does not require the stringent response or changes in abundance of RNA degradation proteins, and can be decoupled from growth status.

RESULTS

mRNA is stabilized as a response to carbon starvation and hypoxic stress in *Mycobacterium smegmatis*

The mRNA pools of *E. coli* and other well-studied bacteria were reported to be globally stabilized during conditions of stress, resulting in increased mRNA half-lives (3-13). Rustad *et al.* reported a similar phenomenon in Mtb under hypoxia and cold shock (3). We sought to establish *M. smegmatis* as a model for study of the mechanistic basis of mRNA stabilization in mycobacteria under stress conditions. We therefore subjected *M. smegmatis* to hypoxia and carbon starvation, and measured mRNA half-lives for a subset of genes by blocking transcription with rifampicin (RIF) and measuring mRNA abundance at multiple time-points using quantitative PCR (qPCR). We used a variation of the Wayne model (55) to produce a gradual transition from aerated growth to hypoxia-induced growth arrest by sealing cultures in vials with defined headspace ratios and allowing them to slowly deplete the available oxygen (Fig. 1A-B).

We tested a set of mRNAs that included leadered and leaderless transcripts, monocistronic and polycistronic transcripts, and transcripts with both relatively short and relatively long half-lives in log phase. We observed that all of the analyzed transcripts had increased half-lives under hypoxia when compared to log phase normoxic cultures and, similarly, transcripts were more stable in carbon starvation than in rich media (Fig. 1C-D). Thus, *M. smegmatis* appears to be a suitable model for investigating the mechanisms of stress-induced mRNA stabilization in mycobacteria. To ensure that the apparent mRNA stabilization was not an artifact of reduced RIF activity in non-growing cells, we confirmed that RIF indeed blocked transcription in hypoxia-arrested *M. smegmatis* (Fig. 1E). We noted that transcripts became progressively more stable as oxygen levels dropped and growth ceased; 40 hours after sealing the vials, mRNA half-lives were too long to reliably measure by our methodology. We sought to focus our studies on the mechanisms that underlie the initial mRNA stabilization process during the transition into hypoxia-induced growth arrest. We therefore conducted most of our subsequent experiments 18-24 hours after sealing the vials, when growth had nearly ceased and transcripts were 9-fold to 25-fold more stable than during log phase. We refer to these conditions as 18 h hypoxia and 24 h hypoxia.

(p)ppGpp does not contribute to mRNA stabilization in hypoxia or carbon starvation

Given recent reports that (p)ppGpp could directly inhibit the enzymatic activity of the exoribonuclease PNPase (42, 43), we wondered whether mRNA stabilization as observed in carbon starvation and hypoxia is regulated by (p)ppGpp in mycobacteria. We obtained a double mutant strain of *M. smegmatis* (56) that lacks both genes implicated in the production of (p)ppGpp ($\Delta rel \Delta sas2$), and compared the mRNA half-lives of a subset of genes to those of wild type mc²155 under hypoxia, log phase normoxia, and carbon starvation. The $\Delta rel \Delta sas2$ strain

had a growth defect during adaptation to hypoxia and carbon starvation (Fig. 2A and 2C), as predicted (57). However, we found no significant decrease in mRNA stabilization in the mutant strain (Fig. 2B and 2D), indicating that the mRNA stabilization observed under hypoxia and carbon starvation is independent from the stringent response. Interestingly, the mutant strain displayed increased mRNA stabilization for a few transcripts under carbon starvation conditions, which could be an indirect consequence of altered transcription rates (see discussion).

Hypoxia-induced mRNA stability is reversible and independent of mRNA abundance

We wondered if the observed stress-induced transcript stabilization could be reversed by restoration of a favorable growth environment. To test this, we prepared 18 h hypoxia cultures, opened the vials and agitated them for 2 min to re-expose the bacteria to oxygen before blocking transcription with RIF and sampling thereafter (Fig. 3A, top). We found that, for all transcripts tested, half-lives were significantly decreased compared to those observed under hypoxia and similar to those observed in log phase (Fig. 3B). While the mechanisms of stress-induced mRNA stabilization are largely unknown, multiple studies have reported inverse correlations between mRNA abundance and half-life in bacteria (3, 8, 53, 54). mRNA abundance was decreased for most transcripts tested in hypoxia-adapted *M. smegmatis*. We therefore considered the possibility that the dramatic increase in mRNA degradation upon re-exposure to oxygen was triggered by a burst of transcription. Indeed, we found increased expression levels for three of five genes tested after two minutes of re-aeration, showing that transcription is rapidly induced upon return to a favorable environment (Fig. 3C). To test the idea that mRNA is destabilized by re-aeration as a consequence of a transcriptional burst and/or increased mRNA abundance, we modified our re-aeration experiment by blocking transcription with RIF one minute prior to re-aeration (Fig. 3A, bottom). Surprisingly, every transcript tested was destabilized by re-aeration despite the absence

of new transcription. For most transcripts, the re-aeration half-lives were indistinguishable regardless of whether RIF was added prior to opening the vials or two minutes after (Fig. 3B). Our results therefore do not support the idea that changes in mRNA abundance alone can explain the mRNA stabilization and destabilization observed in response to changes in energy status. We wanted to further explore if mRNA abundance alone could influence transcript degradation. We obtained a strain encoding *dCas9* and a non-specific sgRNA under control of an ATc-inducible promoter (58) and compared the *dCas9* transcript stability under hypoxia and normoxia after ATc induction or at basal levels. We found that despite a 34-fold transcript upregulation following ATc induction, the half-life of *dCas9* mRNA was not significantly different from the uninduced control in log phase. Under hypoxia, its 28-fold upregulation was associated with an increase in *dCas9* mRNA half-life compared to the no-drug control (Fig. 3D and 3E). Together, our results show that increased mRNA abundance does not necessarily result in a faster decay rate.

mRNA stability is modulated independently of RNase protein levels

Another potential explanation for increased mRNA degradation after re-aeration is the up-regulation of mRNA degradation proteins such as RNase E. To assess the role of a sudden burst in protein levels we used two approaches. First, we constructed strains encoding FLAG-tagged RNase E, cMyc-tagged PNPase, or cMyc-tagged *msmeg_1930* (predicted RNA helicase). We determined protein levels by western blotting in log phase, 18 h hypoxia, and after 18 h hypoxia followed by 2 min re-aeration. Levels of all three of these predicted RNA degradation proteins remained unchanged in the three conditions (Fig. 4A).

Because we do not know all of the proteins that contribute to mRNA degradation in mycobacteria, our second approach was to test the global importance of translation in re-aeration-induced mRNA destabilization. We blocked translation with chloramphenicol (CAM) in 18 h hypoxia cultures and then added RIF. Samples were collected for cultures that remained under hypoxia as well as those that were re-aerated for 2 min (Fig. 4B). For three of the five genes tested, we found that CAM caused increased mRNA stability in hypoxia. This is consistent with CAM's mechanism of action and published work (59-61). CAM inhibits elongation by preventing peptidyl transfer (62-64) and causing ribosomal stalling (65). Global stabilization of mRNA pools has been reported when elongation inhibitors, but not initiation inhibitors, are used for example in log phase cultures of *E. coli* (65) or in yeast (66). We hypothesize that stalled ribosomes may increase mRNA stability by masking RNase cleavage sites. However, despite the stabilization caused by CAM itself, we observed mRNA destabilization in response to re-aeration (Fig. 4C). These results suggest that re-aeration-induced destabilization does not require synthesis of new RNA degradation proteins. Taken together, our data suggest that tuning of protein levels is not the primary explanation for mRNA stabilization during early adaptation to hypoxia.

mRNA stability is modulated in response to changes in metabolic status

The rapidity of mRNA destabilization following re-aeration suggested that mRNA degradation is tightly regulated in response to changes in energy metabolism. We tested this hypothesis by treating log phase cultures with 5 $\mu\text{g}\cdot\text{mL}^{-1}$ bedaquiline (BDQ), a potent inhibitor of the ATP synthase F_0F_1 (67). We used minimal media that contained acetate as the only carbon source (MMA) in order to make the respiratory chain the sole source of ATP synthesis. After 30 min exposure, intracellular ATP levels were reduced by more than 90% compared to cells treated

with vehicle (DMSO), without affecting viability (Fig. 5A and 5B). We then measured half-lives for a set of transcripts under these conditions. mRNA half-lives were dramatically increased in BDQ-treated cells for most of the genes we tested (Fig. 5C), indicating that mRNA degradation rates are rapidly altered in response to changes in energy metabolism status.

We then wondered if we could increase mRNA degradation rates by increasing intracellular ATP levels. To test this, we treated *M. smegmatis* cultures with isoniazid (INH) a pro-drug that interferes with the synthesis of mycolic acids, and also leads to an accumulation of intracellular ATP due to increased oxidative phosphorylation (68). We exposed *M. smegmatis* to 500 $\mu\text{g}\cdot\text{mL}^{-1}$ INH for 6.5 hours to confirm that we had achieved bacteriostasis (*M. smegmatis* doubling time in MMA media is ~six hours). As shown in Fig. 5D, INH caused a dramatic increase in intracellular ATP after 6.5 h without affecting cell viability (Fig. 5E). Remarkably, mRNA half-lives were significantly decreased in response to INH (Fig. 5F). To our knowledge, this is the first report of bacterial mRNA being destabilized rather than stabilized in response to a growth-impairing stressor. Our results indicate that mRNA stability is regulated not in response to growth status per se, but rather to energy metabolism. Although we interpreted ATP levels as a reflection of metabolic status in our INH and BDQ assays, the coupling between mRNA degradation and metabolic status does not appear to be mediated by ATP directly. We measured ATP levels in cultures during the transition to hypoxia-induced growth arrest, and found that although ATP levels ultimately decrease in hypoxia as has been reported elsewhere (69, 70), mRNA stabilization precedes the drop in ATP levels (Fig. 5G).

DISCUSSION

246 Stressors that cause bacteria to slow or stop growth are typically associated with increased
247 mRNA stability (3-9, 11-13). Many of these same stressors reduce energy availability (69, 70),
248 requiring reductions in energy consumption and optimization of resource allocation. We
249 speculate that the decreased mRNA turnover that accompanies such conditions may be an energy
250 conservation mechanism. For Mtb, hypoxia can lead to generation of bacterial subpopulations
251 with varying degrees of antibiotic tolerance (71-73), facilitating bacterial survival and the
252 acquisition of drug resistance-conferring mutations. Understanding the mechanisms that support
253 the transitions into non-growing states, and subsequent survival in these states, is therefore a
254 priority.

255 The transcriptome of Mtb has been shown to be stabilized under cold shock and hypoxia (3).
256 Here, we found that *M. smegmatis* also dramatically stabilized its mRNA in response to carbon
257 starvation and hypoxia. For the first time, to our knowledge, we tested the speed at which this
258 stabilization is reversed in mycobacteria upon restoration of energy availability. Remarkably,
259 mRNAs are rapidly destabilized within minutes of re-aeration of hypoxic cultures, suggesting
260 that tuning of mRNA degradation rates is an early step in the response to changing energy
261 availability.

262 The most straightforward explanation for stress-induced mRNA stabilization would seem to be
263 downregulation of the mRNA degradation machinery. Indeed, RNase E is downregulated at the
264 transcript level under hypoxia, and abundance of cleaved RNAs is reduced (74). However, we
265 found that protein levels were unchanged for RNase E and two other proteins predicted to be
266 core components of the mRNA degradation machinery. This is largely consistent with what was
267 reported for Mtb in a quantitative proteomics study (37), although in that case there was an
268 apparent reduction in levels of an RNA helicase. To address this question in a more agnostic

269 fashion, we tested the importance of translation for transcript destabilization upon re-exposure of
270 hypoxic cultures to oxygen. However, re-aeration triggered increased transcript degradation even
271 in the absence of new protein synthesis. Regulation of degradation protein levels therefore does
272 not appear to contribute to mRNA stabilization during the initial response to energy stress.
273 However, we found that upon longer periods of hypoxia, transcripts were stabilized to a greater
274 extent than what we observed 18 hours after sealing the vials. This suggests that mRNA
275 stabilization progressively increases, and may involve multiple mechanisms. As this work
276 focused on the initial transition into hypoxia-induced growth arrest, we cannot discount the
277 possibility that downregulation of the RNA degradation machinery is important for further
278 mRNA stabilization in later hypoxia stages.

279 Interestingly, we found greater mRNA stabilization in hypoxic cultures treated with CAM. This
280 may result from stalled ribosomes (62, 64) masking RNase cleavage sites. Furthermore, the burst
281 of transcription upon re-aeration is blocked by the presence of CAM, causing up to a four-fold
282 decrease in transcript abundance in the CAM treated cultures when compared to the vehicle
283 treated cultures. This is consistent with the idea that transcription and translation are physically
284 coupled, and blocking translation therefore prevents RNA polymerase from efficiently carrying
285 out transcript elongation, as was reported for *E. coli* (75-79). The results obtained from the Δrel
286 $\Delta sas2$ strain are also consistent with the idea that the presence of ribosomes affects mRNA
287 stability. Under carbon starvation this strain had rRNA levels three-fold higher than the WT
288 strain, consistent with the known role of (p)ppGpp in downregulating ribosome biogenesis (80-
289 82). Interestingly, some transcripts were hyperstabilized in the $\Delta rel \Delta sas2$ strain under carbon
290 starvation, showing virtually no degradation (Fig. 2D). We speculate that the observed mRNA
291 hyperstabilization could be caused by increased ribosome abundance, resulting in augmented

292 mRNA-ribosome associations that ultimately protect transcripts from RNases. Alternatively, the
293 increased abundance of rRNA could protect mRNA indirectly by providing alternative targets
294 that compete for interaction with RNases (65).

295 Transcript abundance has been found to be inversely correlated with mRNA stability in
296 exponentially growing bacteria (3, 8, 53, 54, 83), and experimental manipulation of transcription
297 rates of subsets of genes affected their degradation rates (3, 54). Together, these studies suggest
298 that high rates of transcription inherently increase degradation rates. We report here that during
299 oxygen depletion transcript levels are reduced in *M. smegmatis*, which led us to ask if increased
300 transcript half-lives under stress are a direct result of reduced mRNA levels. However, our data
301 are inconsistent with this idea; mRNA is rapidly destabilized upon re-aeration even in the
302 absence of new transcription. We note that one study reported a weak positive correlation
303 between mRNA abundance and stability in log phase *E. coli* (12), while another reported mRNA
304 abundance to be positively correlated with stability in carbon-starved *Lactococcus lactis* (8).
305 Together, these observations and our own suggest that the relationship between mRNA stability
306 and abundance is not yet fully understood and may be fundamentally different in growth-arrested
307 bacteria.

308 The rapid reversibility of hypoxia-induced mRNA stabilization suggests that mRNA decay and
309 energy metabolic status are closely linked. Consistent with this, we have shown that drug-
310 induced energy stress causes mRNA stabilization, while mRNA decay is increased by a drug that
311 induces a hyperactive metabolic state. To our knowledge this is the first demonstration that the
312 rate of bacterial mRNA degradation can be decoupled from growth rate, and suggests that
313 mRNA decay is controlled by energy status rather than growth rate per se. The mechanism by
314 which energy status and mRNA decay are coupled remains elusive; the stringent response is not

required, and the stabilization of mRNA during adaptation to hypoxia precedes a decrease in ATP levels. Our data are consistent with two general (non-exclusive) models: mRNA decay could be regulated by protection of transcripts from RNase attack, and/or by direct regulation of the activity of the RNases. Possible explanations that fall within one or both of these frameworks include changes in ribosome occupancy, the presence of other RNA-binding proteins, regulation of the subcellular localization of mRNAs and/or the RNA degradation machinery, and altered degradosome composition. These possibilities should be investigated in future work.

METHODS

Strains and culture conditions

Mycobacterium smegmatis strain mc²155 or derivatives (Table 1) were grown in *rich medium*, Middlebrook 7H9 with ADC (Albumin Dextrose Catalase, final concentrations 5 g·L⁻¹ BSA fraction V [BSA], 2 g·L⁻¹ dextrose, 0.85 g·L⁻¹ NaCl, and 3 mg·L⁻¹ catalase), 0.2% glycerol and 0.05% Tween 80 at 200 rpm and 37°C to an OD₆₀₀ of ~0.8, unless specified otherwise. For hypoxic cultures, we modified the Wayne model (55). Bacteria were cultured in 30.5 x 58 mm serum bottles (Wheaton, 223687, 20 mL) using *rich medium* and an initial OD₆₀₀=0.01. Bottles were sealed with a vial crimper (Wheaton, W225303) using rubber stoppers (Wheaton, W224100-181) and aluminum seals (Wheaton, 224193-01). Oxygen levels were qualitatively monitored using methylene blue.

For carbon starvation cultures, cells were grown to log phase (OD₆₀₀=0.8) in *rich medium*, pelleted and rinsed three times with *carbon starvation medium* (Middlebrook 7H9 with 5 g·L⁻¹

336 BSA, 0.85 g·L⁻¹ NaCl, 3 mg·L⁻¹ catalase, and 0.05 % Tyloxapol) at 4°C, then resuspended in
337 *carbon starvation medium* to OD₆₀₀= 0.8 and incubated at 200 rpm, 37°C.

338 The RNase E-tagged strain (SS-M_0296) was built using a two-step process. Plasmid pSS250
339 was derived from pJM1 (84) and contained 1 kb of the sequence upstream and downstream of
340 the *rne* (msmeg_4626) start codon, with the sequence encoding 6xHis-3xFLAG-TEV-4xGly
341 inserted after the start codon. Constructs were built using NEBuilder HiFi (E2621). Integrants
342 were selected with 200 µg·mL⁻¹ hygromycin and confirmed by sequencing. Counter-selection
343 with 15% sucrose was followed by PCR screening to identify isolates that underwent second
344 crossovers resulting in loss of the plasmid and retention of tagged *rne*.

345 The PNPase-tagged strain (SS-M_0412) was built by inserting a second copy of *pnp*
346 (msmeg_2656) with an N-terminal c-Myc-4xGly and its predicted native promoter and 5' UTR
347 at the Giles phage integration site (plasmid pSS282) into strain SS-M_0296. The RNA helicase-
348 tagged strain (SS-M_0416) was constructed in a similar way but using a C-terminal 4xGly-c-
349 Myc tag on msmeg_1930 (plasmid pSS285).

350 **RNA extraction and determination of mRNA stability**

351 Biological triplicate cultures were treated with rifampicin (RIF) to a final concentration of 150
352 µg·mL⁻¹ to halt transcription and RNA was extracted at various time-points thereafter. For
353 exponential and carbon starvation cultures, 7 mL were collected per replicate and time-point and
354 snap-frozen in liquid nitrogen (LN2). For hypoxic samples, degassed RIF was injected using a
355 30G needle, and all samples were sacrificially collected per time-point and replicate (7 mL) and
356 snap-frozen in LN2 within 6 seconds of unsealing.

357 Samples were stored at -80°C and thawed on ice immediately before RNA extraction. Cells were
 358 pelleted at 4°C , resuspended in 1 mL TRIzol (Invitrogen), transferred to 2 mL disruption tubes
 359 (OPS Diagnostics 100 μm zirconium lysing matrix, molecular grade), and lysed using a
 360 FastPrep-24 5G (MP) (3 cycles of $7\text{ m}\cdot\text{s}^{-1}$ for 30 s, 2 min on ice between cycles). 300 μL
 361 chloroform was added, samples centrifuged 15 min at $21,130\times g$ and 4°C , and RNA recovered
 362 from the aqueous layer and purified with a Direct-zol RNA MiniPrep kit according to the
 363 manufacturer's instructions with in-column DNase treatment. Agarose gels were used to verify
 364 RNA integrity.

365 For cDNA synthesis, 600 ng of total RNA were mixed with 0.83 μL 100 mM tris pH 7.5 and
 366 0.17 μL 3 $\text{mg}\cdot\text{mL}^{-1}$ Random Primers (NEB) in 5.25 μL , denatured at 70°C for 10 min and snap-
 367 cooled. Reverse transcription was performed for 5 hours at 42°C using 100 U ProtoScript® II
 368 Reverse Transcriptase (NEB), 10 U RNase Inhibitor (Murine, NEB), 0.5 mM each dNTP mix
 369 and 5 mM DTT in a final volume of 10 μL . RNA was degraded with 5 μL each 0.5 mM EDTA
 370 and 1 N NaOH at 65°C for 15 min, followed by 12.5 μL of 1 M Tris-HCl pH 7.5. cDNA was
 371 purified using the MinElute PCR Purification Kit (Qiagen) according to the manufacturer
 372 instructions. mRNA abundance (A) over time (t) was determined for different genes (primers in
 373 Table 2) by quantitative PCR (qPCR) using iTaq SYBR Green (Bio-Rad) with 400 pg of cDNA
 374 and 0.25 μM each primer in 10 μL reactions, with 40 cycles of 15 s at 95°C and 1 min at 61°C
 375 (Applied Biosystems 7500). Abundance was expressed as the $-C_T$ (reflecting the $\log_2 A(t)$).
 376 Linear regression was performed on $-C_T$ values versus time where the negative reciprocal of the
 377 best-fit slope estimates mRNA half-life (see supplemental materials, Text S1 and Fig. S1). In
 378 many cases the decay curves were biphasic, with a rapid period of decay followed by a period of

slow or undetectable decay. In these cases, only the initial, steeper slope was used for calculation of half-lives.

mRNA stability during re-aeration and translational inhibition

Translation was halted by $150\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ chloramphenicol, rifampicin was added 1 min later, and samples collected starting 1 min after that. For re-aeration experiments, 18 h hypoxia cultures were opened, the contents transferred to 50 mL conical tubes, and triplicate samples taken 2, 7, 12, 17, and 32 min after opening the bottles and snap-frozen in LN2. Rifampicin was added either 1 min before (transcription inhibition during hypoxia) or 2 min after opening the bottles (transcription inhibition after re-aeration).

Bedaquiline and isoniazid treatments

Cultures were grown to $\text{OD}_{600} \sim 1.0$ in *rich medium*, rinsed twice in *Minimal Media Acetate wash* (final concentrations: $0.5\text{ g}\cdot\text{L}^{-1}$ L-asparagine, $1\text{ g}\cdot\text{L}^{-1}$ KH_2PO_4 , $2.5\text{ g}\cdot\text{L}^{-1}$ Na_2HPO_4 , $0.5\text{ g}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, $0.5\text{ mg}\cdot\text{L}^{-1}$ CaCl_2 , $0.1\text{ mg}\cdot\text{L}^{-1}$ ZnSO_4 , 0.1% CH_3COONa , 0.05% tyloxapol, pH 7.5) at 4°C , resuspended in Minimal Media Acetate with ferric ammonium citrate (MMA, *Minimal Media Acetate wash* + $50\text{ mg}\cdot\text{L}^{-1}$ ferric ammonium citrate) to $\text{OD}_{600}=0.07$, and grown for 24 hours to $\text{OD}_{600} \sim 0.8$. To remove the extracellular ATP, 30 minutes before drug treatment cells were pelleted and rinsed in pre-warmed *Minimal Media Acetate wash*, resuspended in pre-warmed MMA, and returned to the incubator. Bedaquiline (BDQ), isoniazid (INH), or their vehicles were added to final concentrations of $5\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ or $500\text{ }\mu\text{g}\cdot\text{mL}^{-1}$, respectively. Samples were taken 30 min after adding BDQ, or 6.5 h after adding INH for half-life and ATP determination.

400 For half-life measurements, BDQ cultures were sampled 0, 3, 6, 9, 12, 15 and 21 min after
401 adding RIF and INH cultures were sampled 0, 4, 8 and 12 min after adding RIF. RNA
402 extractions were performed as above with the following modifications: cell disruption was
403 performed using 2 mL tubes prefilled with Lysing Matrix B (MP) and 3 cycles of 10 m·s⁻¹ for 40
404 s; RNA was recovered from the aqueous layer by isopropanol precipitation and resuspension in
405 H₂O; samples were treated with 5 U of TURBO™ DNase (Ambion) in presence of 80 U of
406 RNase Inhibitor, Murine (NEB) for 1 hour at 37°C with agitation. RNA was purified with an
407 RNeasy Mini Kit (Qiagen) according to the manufacturer's specifications.

408 **Intracellular ATP estimation**

409 ATP was estimated by BacTiter-Glo (Promega). For BDQ or INH treatments, 1 mL of culture
410 was pelleted at ~21°C for 1 min at 21,130 x g, the supernatant removed and cells resuspended in
411 1 mL of pre-warmed MMA containing BDQ, INH, or vehicle to match the prior treatment
412 condition. Immediately after, 20 µL samples were transferred to a white 384-well plate (Greiner
413 bio-one) containing 80 µL of BacTiter-Glo reagent and mixed for 5 minutes at room
414 temperature. Luminescence was measured in a VICTOR³ plate reader (PerkinElmer)
415 (intracellular ATP). We included controls for the supernatant collected (extracellular ATP),
416 media + drug/vehicle (background), and ATP standards for constructing standard curves.

417 To estimate intracellular ATP in normoxia and hypoxia cultures, 20 µL samples were collected
418 at 37°C and immediately combined with the reagent to measure total ATP (intracellular +
419 extracellular). From the same cultures, 1 mL samples were syringe-filtered (PES 0.2 µm) and the
420 filtrate combined with the reagent to measure extracellular ATP. Luminescence was measured as
421 above. Intracellular ATP was calculated by subtracting the extracellular ATP values from the

total ATP values. Hypoxia samples were sacrificially harvested per time-point/replicate and combined with the reagent in <6 seconds.

AUTHOR CONTRIBUTIONS

DVB and SSS conceived and designed the experiments. DVB and YZ performed the experiments. LGZ performed part of the experiments in Fig. 3. DVB, TA and SSS analyzed the data. DVB and SSS wrote the manuscript.

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FIGURE LEGENDS

Figure 1

Transcript half-lives are increased in response to hypoxia and carbon starvation stress. (A)

Growth kinetics for *M. smegmatis* under hypoxia using a variation of the Wayne model (55), showing OD stabilization at 18-24 hours. Oxygen depletion was assessed qualitatively by methylene blue discoloration. (B) *M. smegmatis* was sealed in vials to produce a hypoxic environment, at 18 hours transcription was inhibited with RIF and samples collected thereafter. Transcript half-lives for the indicated genes were measured for *M. smegmatis* mc²155 after blocking transcription with 150 $\mu\text{g}\cdot\text{mL}^{-1}$ RIF. RNA samples were collected (C) during log phase normoxia, and hypoxia (18 hours after closing the bottles); or (D) during log phase in 7H9 supplemented with ADC, glycerol, and Tween 80 (rich media) or 7H9 with Tyloxapol only (carbon starvation, 24 hours). Degradation rates were compared using linear regression ($n=3$), and half-lives were determined by the reciprocal of the best-fit slope. Error bars: 95% CI. *** $p<0.001$; **** $p<0.0001$. When a slope of zero was included in the 95% CI (indicating no degradation), the upper limit for half-life was unbounded, indicated by a clipped error bar with a double line. (E) RIF blocks overexpression of an ATc-inducible gene (*rraA*) in hypoxic cultures. 40 h after sealing bottles, cultures were treated with 50 $\text{ng}\cdot\text{mL}^{-1}$ ATc and/or 150 $\mu\text{g}\cdot\text{mL}^{-1}$ RIF or the drug vehicle (DMSO) for 1 h. Expression levels (qPCR) are displayed relative to no drugs (DMSO) treatment. ATc, RIF and DMSO solutions were degassed prior to addition. Error bars: SD.

Figure 2

Transcript stabilization in hypoxia and carbon starvation is not dependent on the stringent

response. (A) Growth kinetics for *M. smegmatis* mc²155 (WT) and Δrel , $\Delta sas2$ strains cultured

in 7H9 in flasks sealed at time 0. (B) Transcript half-lives for a set of genes 24 hours after

sealing the hypoxia bottles (arrow in A). RNA samples were collected after blocking

transcription with 150 $\mu\text{g}\cdot\text{mL}^{-1}$ RIF (degassed). (C) Bacteria were grown to log phase in 7H9

supplemented with ADC, glycerol, and Tween 80, then transferred to 7H9 supplemented with

Tyloxapol only at time 0. (D) Transcript stability for a set of genes 22 hours after transfer to

carbon starvation media (arrow in C). In A and C, the mean and SD of triplicate cultures is

shown. In B and D, half-lives were compared using linear regression analysis ($n=3$). Error bars:

95% CI. **** $p<0.0001$, n.s. $p>0.05$. In cases where no degradation was observed or when the

upper 95% CI limit was unbounded, the bar or upper error bar were clipped, respectively.

Figure 3

Hypoxia-induced mRNA stability is reversible and independent of mRNA abundance. (A)

M. smegmatis was sealed in vials for 18 hours to produce a hypoxic environment, then re-

exposed to oxygen for two minutes before transcription was inhibited RIF (top) or injected with

RIF one minute prior to opening the vials and re-exposing to oxygen (bottom). (B) Transcript

half-lives for a set of genes are displayed for log phase normoxia cultures, hypoxia (18 h), and

re-aeration with RIF added either before or after opening the vials. Half-lives were compared by

linear regression analysis ($n=3$). (C) Expression levels of transcripts in hypoxia (18 h) or 2 min

re-aeration relative to the expression levels in log phase normoxia cultures (percentage). Error

bars: SD. (D) Expression levels of transcripts in hypoxia (18 h) or log phase normoxia after

being treated with 200 ng·mL⁻¹ ATc for 1 h or 10 min, respectively, to induce *dCas9* overexpression, relative to the expression levels in a H₂O vehicle treatment (percentage). Error bars: SD. (E) Transcript half-lives for *dCas9* and *sigA* for log phase normoxia and hypoxia (18 h) after induction of *dCas9* with ATc or vehicle treatment as shown in D. In B and E, degradation rates were compared using linear regression ($n=3$), and half-lives were determined by the reciprocal of the best-fit slope. Error bars: 95% CI. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$, n.s. $p>0.05$. RIF added to hypoxic cultures was degassed prior to addition.

Figure 4

mRNA stability is regulated independently of degradation protein levels. (A) Western blotting for FLAG-tagged RNase E, and c-Myc-tagged PNPase or RNA helicase (*msmeg_1930*) in *M. smegmatis* in log phase normoxia, hypoxia (18 h), and 2 min re-aeration. Samples were normalized to total protein level, which were similar on a per-OD basis in all conditions. (B) Translation was inhibited in hypoxic cultures by 150 µg·mL⁻¹ CAM 1 min before adding 150 µg·mL⁻¹ RIF. RNA was harvested at time points beginning 2 min after adding CAM. (C) Transcript half-lives for samples from hypoxic cultures with the drug vehicle (ethanol), hypoxic cultures after translation inhibition, and 2 min re-aeration after translation inhibition. Degradation rates were compared using linear regression ($n=3$), and half-lives were determined by the reciprocal of the best-fit slope. Error bars: 95% CI. n.s., $p>0.05$, * $p<0.05$, *** $p<0.001$, **** $p<0.0001$. Drugs and drug vehicles added to the hypoxic cultures were degassed prior to addition.

Figure 5

mRNA stability is modulated in response to changes in metabolic status. (A) *M. smegmatis* was cultured in MMA media for 22 hours to OD₆₀₀ 0.8 before being treated with 5 µg·mL⁻¹ BDQ or the vehicle (DMSO) for 30 min. Intracellular ATP was determined using the BacTiter-Glo kit. (B) Growth kinetics for *M. smegmatis* from panel A in presence of BDQ. (C) Transcript half-lives for a sub-set of transcripts collected during intracellular ATP depletion (30 min with BDQ) or at the basal levels (30 min with DMSO). (D) As in panel A, but for *M. smegmatis* treated with 500 µg·mL⁻¹ INH or the vehicle (H₂O) for 6.5 hours. (E) Growth kinetics for *M. smegmatis* from panel D in presence of INH. (F) Transcript half-lives for a sub-set of transcripts after 6.5 h of INH or vehicle treatment. (G) Growth kinetics for *M. smegmatis* transitioning into hypoxia, and intracellular ATP levels at different stages. Bottles were sealed at time 0. The dotted line represents the time at which transcript stability analysis were made for the hypoxia (18 h) condition for Figures 1-4. In C and F, half-lives were compared using linear regression analysis ($n=3$). Error bars: 95% CI. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. ATP was measured in biological triplicate cultures and is representative of at least two independent experiments.

TABLES

TABLE 1

Strains used and sources

Strain	Characteristics	Reference or source
mc ² 155	<i>M. smegmatis</i> , WT	(85)
SS-M_0072	mc ² 155 derivative transformed with plasmid pSS162, containing an ATc-inducible copy of <i>rraA</i> .	This work
SS-M_0296	mc ² 155 in which the native copy of RNase E (<i>rne</i>) is N-terminally tagged with 6xHis-3xFLAG-TEV-4xGly linker (CACCACCACCACCACCACGATTACAAGGATCACGATGGCGATTACAAGGATCATGACATCGACTATAAGGACGATGACGATAAGGAGAACCTGTACTTCCAGGGCGGCGGCGGC).	This work
SS-M_0412	SS-M_0296 derivative containing a second copy of PNPase (<i>msmeg_2656</i>) with its predicted native promoter and 5' UTR, and N-terminally tagged with c-Myc-4xGly-linker (GAGCAGAAGCTGATCTCGGAAGAGGACCTCGGCGGCGGCGGC) contained on Giles-integrating plasmid pSS282 (Hyg ^R).	This work
SS-M_0416	SS-M_0296 derivative containing a second copy of RNA helicase (<i>msmeg_1930</i>) with its predicted native promoter and 5' UTR, and C-terminally tagged with 4x Gly linker-c-Myc (GGCGGCGGCGGCGAGCAGAAGCTGATCTCGGA) contained on a Giles-integrating plasmid pSS285 (Hyg ^R).	This work
Δrel_{Msm}	mc ² 155 derivative, $\Delta rel \Delta sas2$	(56)
SS-M_0203	mc ² 155 derivative transformed with plasmid pJR962, containing an ATc regulated <i>dCas9</i> .	(58)

TABLE 2

Primers for qPCR

Primer name	Gene	Directionality	Sequence 5' → 3'
SSS903	<i>atpB</i> (msmeg_4942)	Forward	TGTTTCGTGTTTCGTCTGCTAC
SSS904	<i>atpB</i> (msmeg_4942)	Reverse	CGGCTTGGCGAGTTCTT
SSS909	<i>atpE</i> (msmeg_4941)	Forward	GGGTAACGCGCTGATCTC
SSS910	<i>atpE</i> (msmeg_4941)	Reverse	GAAGGCCAGGTTGATGAAGTA
SSS1241	<i>dCas9</i>	Forward	GACAAGTCGAAGTTCCTGATGTA
SSS1242	<i>dCas9</i>	Reverse	GATCTGCTTGTTTCGGGTAGTT
SSS537	<i>esxB</i> (msmeg_0065)	Forward	GGTGAGGACACAGGGAAATAAG
SSS538	<i>esxB</i> (msmeg_0065)	Reverse	CGGAGATGCGCTCGAAAT
SSS856	<i>katG</i> (msmeg_6384)	Forward	GGCCCAATCAGCTCAATCT
SSS857	<i>katG</i> (msmeg_6384)	Reverse	CGGACCGGTAGTCGAAATC
SSS706	<i>rnj</i> (msmeg_2685)	Forward	TCATCCTCTCATCGGGTTTC
SSS707	<i>rnj</i> (msmeg_2685)	Reverse	TTCGCGCTCAACCTTCT
SSS697	<i>rraA</i> (msmeg_6439)	Forward	AACTACGGCGGCAAGAT
SSS698	<i>rraA</i> (msmeg_6439)	Reverse	GTCGAGAGGATCGACTTCAG
JR273 (58)	<i>sigA</i> (msmeg_2758)	Forward	GACTACACCAAGGGCTACAAG
JR274 (58)	<i>sigA</i> (msmeg_2758)	Reverse	TTGATCACCTCGACCATGTG

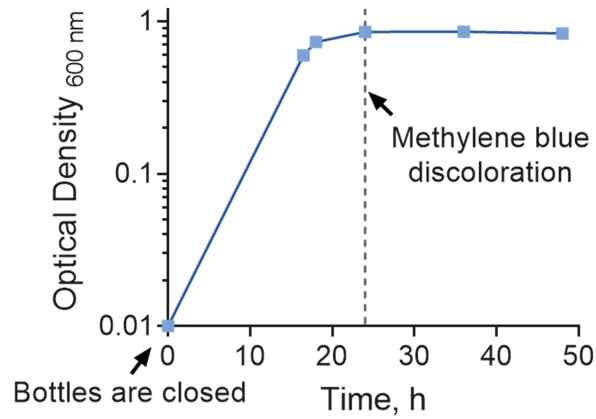
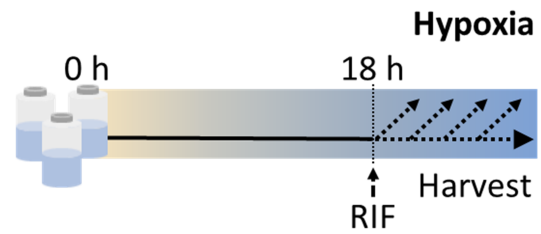
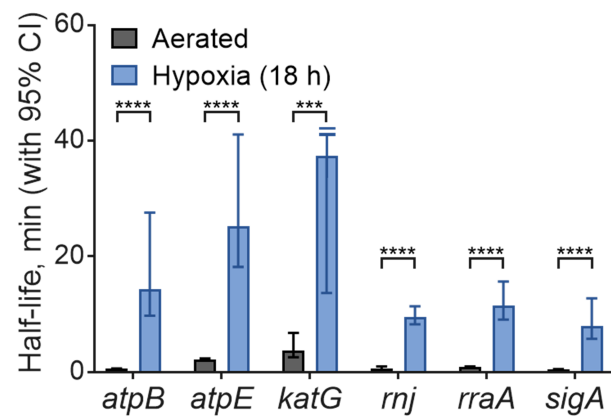
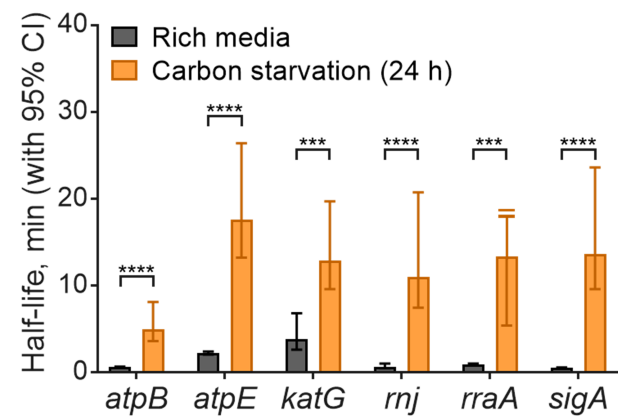
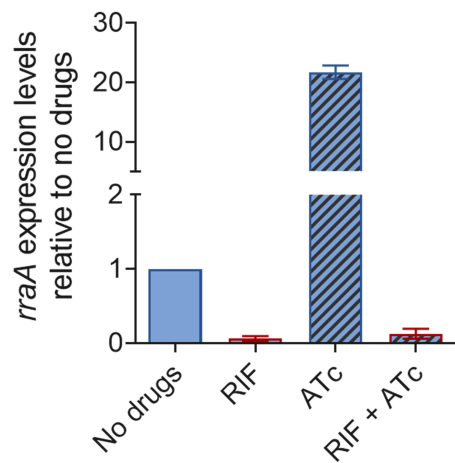
SUPPLEMENTAL METHODS

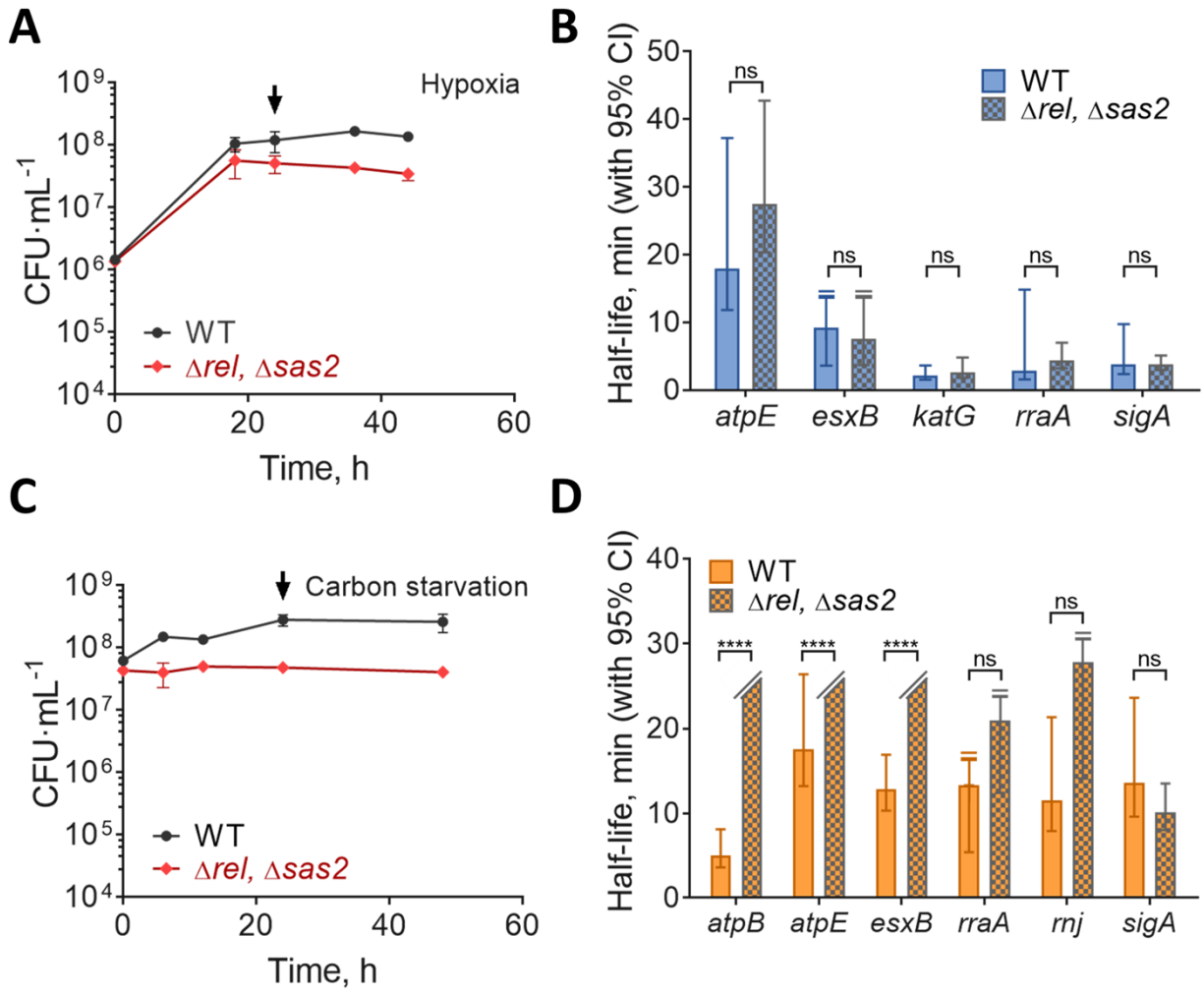
The supplemental methods (Text S1) provide additional detail about the methodology used to measure mRNA half-lives.

SUPPLEMENTAL FIGURE LEGENDS

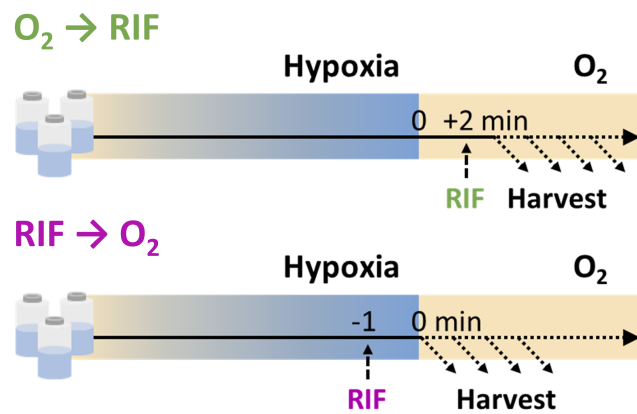
Supplemental Figure 1

mRNA decay curves for an example gene, *rraA*. x axis denotes time after transcription was blocked by addition of RIF. (A) $-C_T$ versus time data for *rraA* giving a half-life estimate of 0.935 minutes. (B) Estimated mRNA abundance for *rraA* relative to the time of RIF addition, giving a half-life estimate of 0.935 minutes.

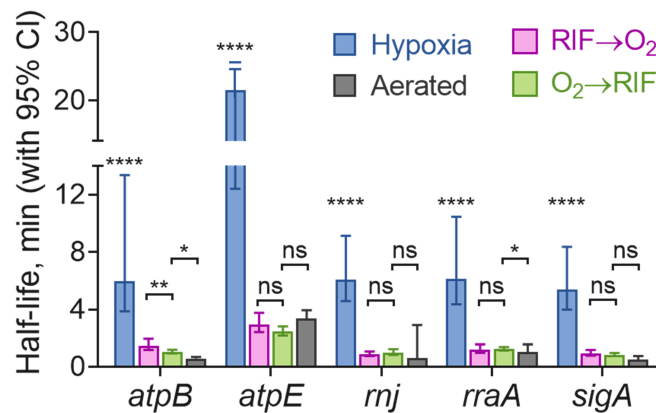
A**B****C****D****E**



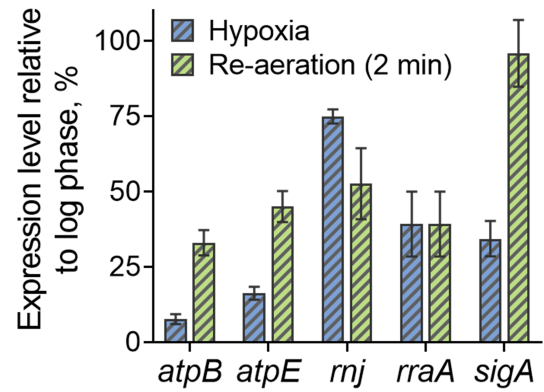
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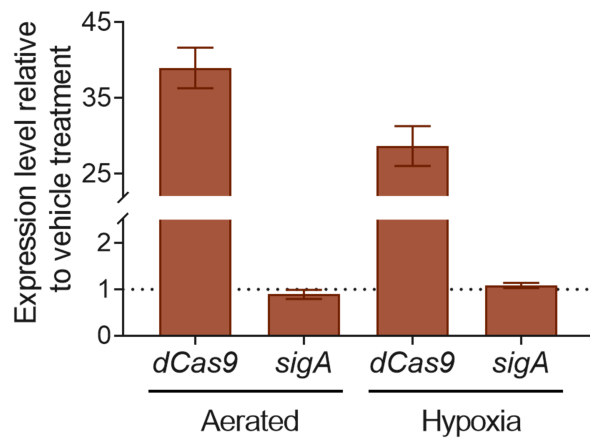
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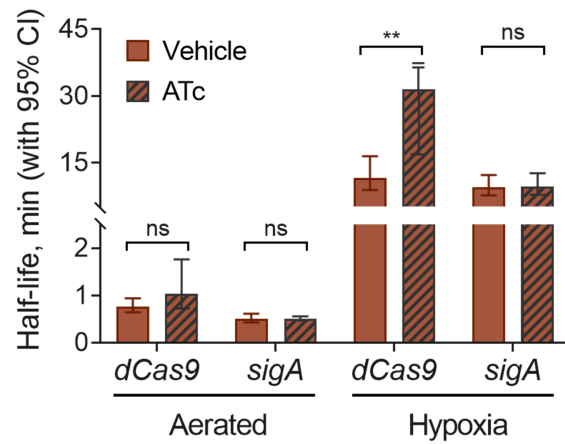
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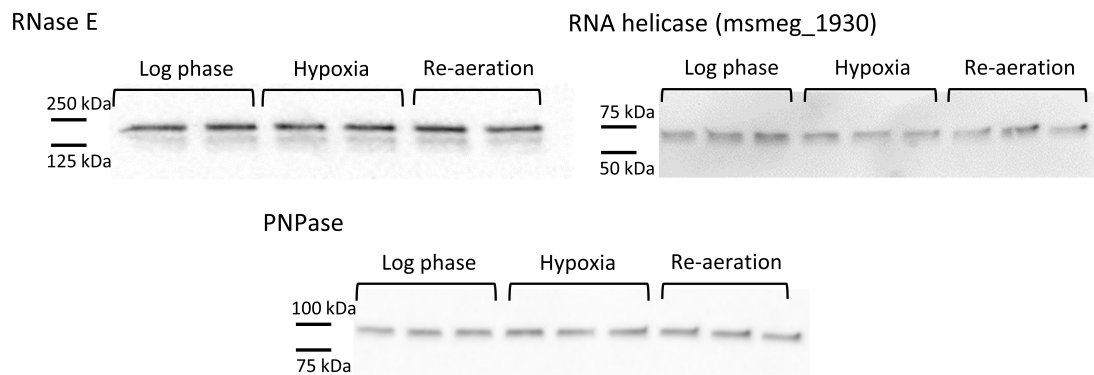


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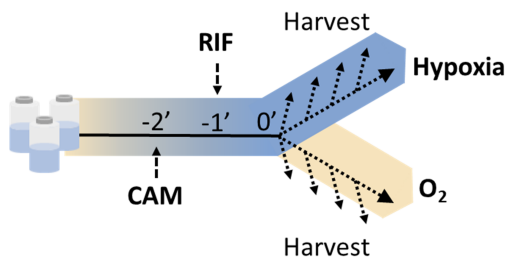


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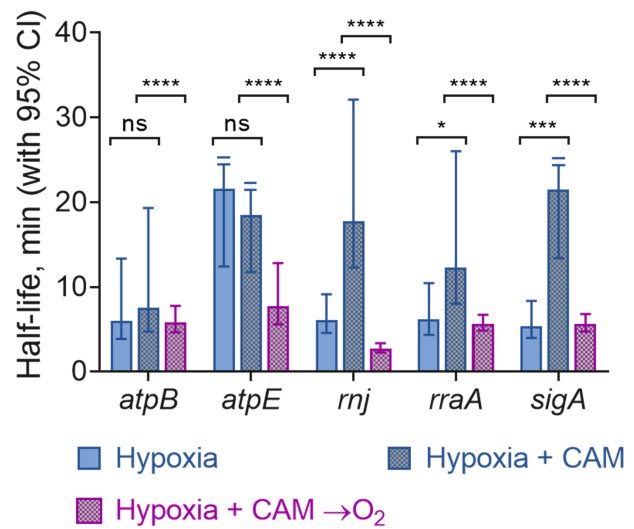
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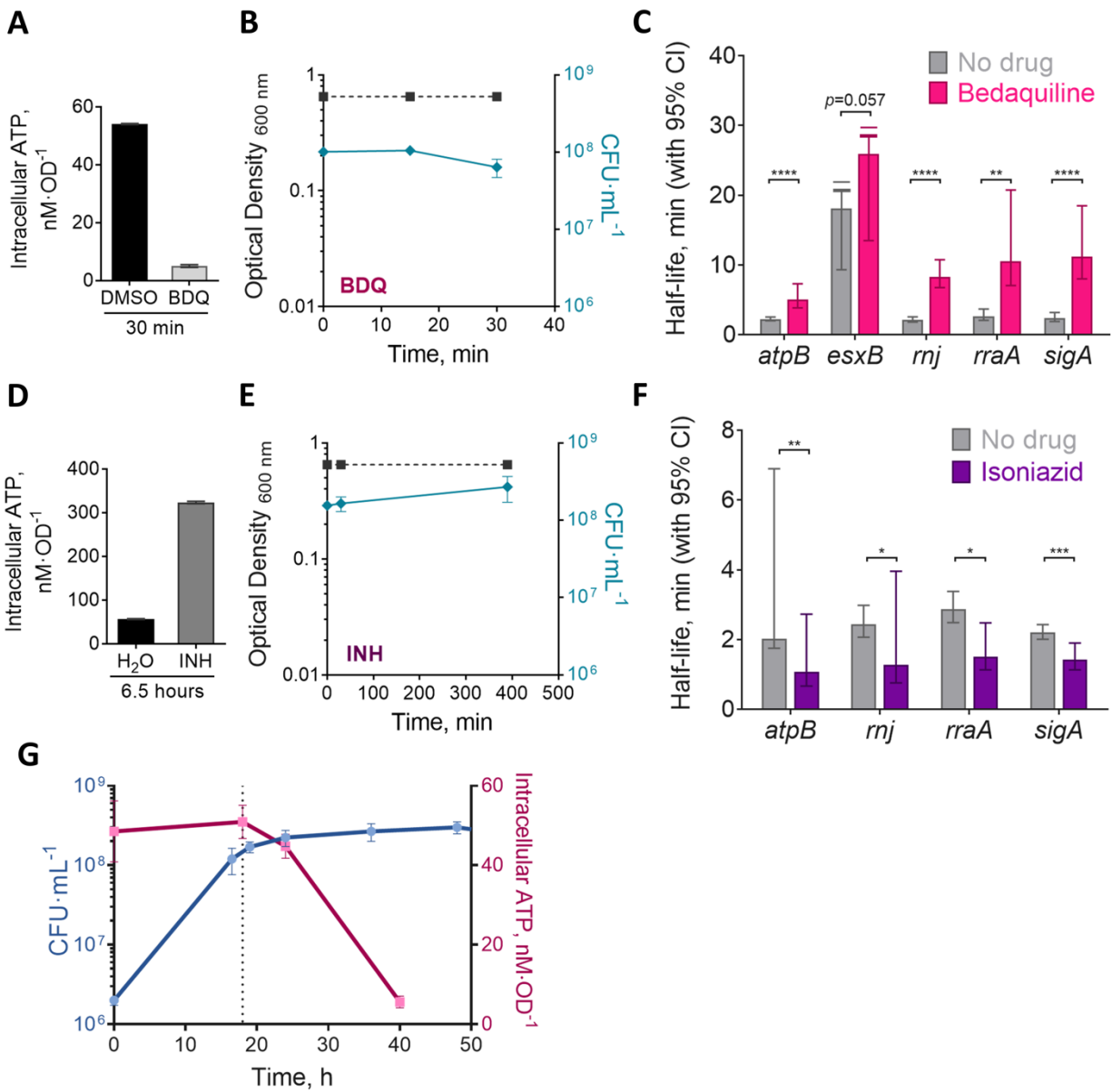
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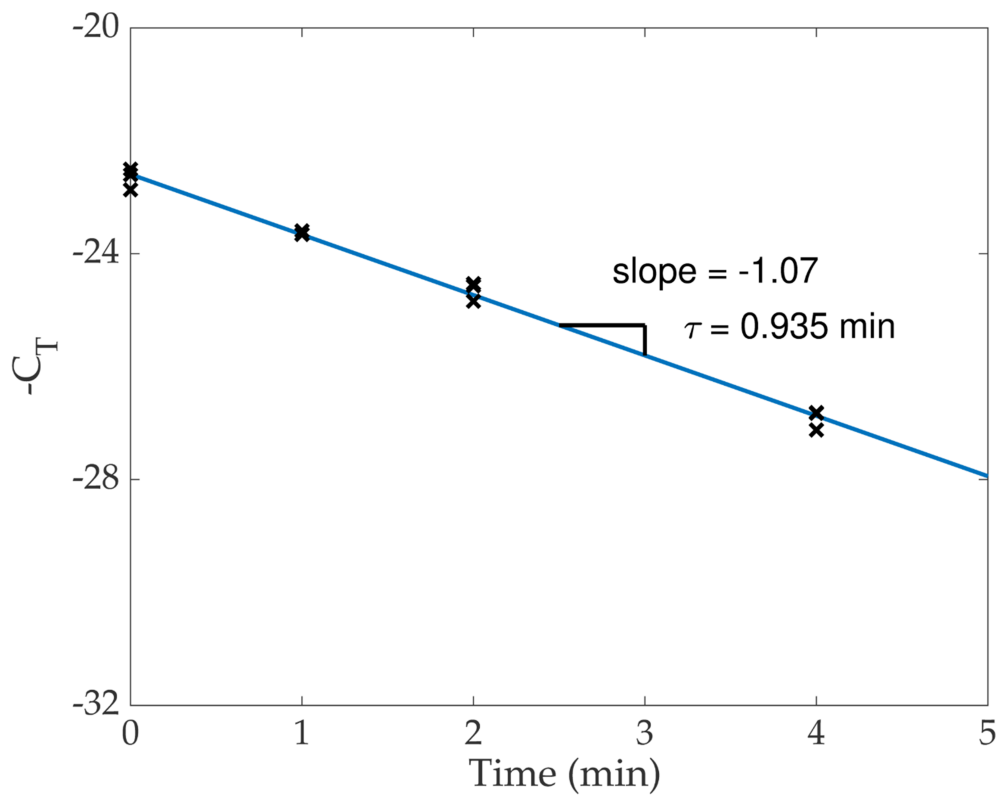
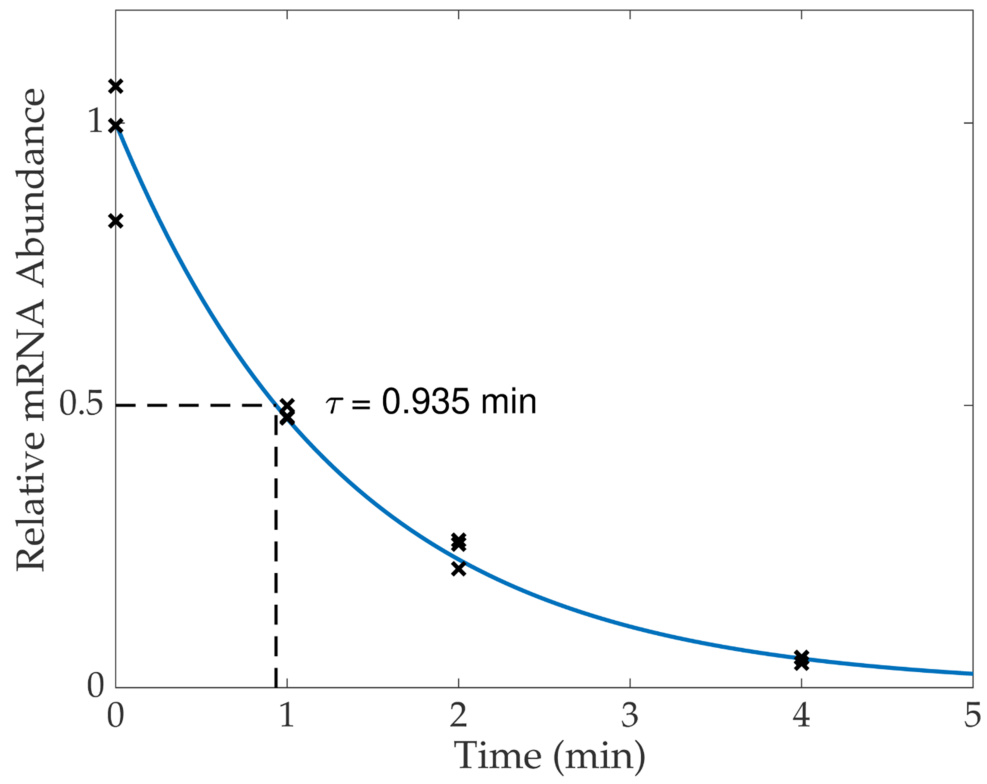
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A**B**

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