Mycobacterial HelD is a nucleic acids-clearing factor for RNA polymerase

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RNA synthesis is central to life, and RNA polymerase (RNAP) depends on accessory factors for recovery from stalled states and adaptation to environmental changes. Here, we investigated the mechanism by which a helicase-like factor HelD recycles RNAP. We report a cryo-EM structure of a complex between the Mycobacterium smegmatis RNAP and HelD. The crescent-shaped HelD simultaneously penetrates deep into two RNAP channels that are responsible for nucleic acids binding and substrate delivery to the active site, thereby locking RNAP in an inactive state. We show that HelD prevents non-specific interactions between RNAP and DNA and dissociates stalled transcription elongation complexes. The liberated RNAP can either stay dormant, sequestered by HelD, or upon HelD release, restart transcription. Our results provide insights into the architecture and regulation of the highly medically-relevant mycobacterial transcription machinery and define HelD as a clearing factor that releases RNAP from nonfunctional complexes with nucleic acids.
smoothly functioning transcription machinery is essential for maintaining the physiologically relevant levels of gene products and adequate changes in transcription are necessary for cell survival when the environment changes. In bacteria, transcription is executed by a single enzyme, DNA-dependent RNA polymerase (RNAP; composition of the core enzyme: α₂ββ′ω). The RNAP core is capable of transcription elongation and termination but not initiation. To initiate, a σ factor is required to form a holoenzyme that recognizes species-specific promoter sequences. The primary σ factor is termed σE in E. coli and σ70 in most other species.

The two largest subunits, β and β′, held together by the α dimer, form a crab-claw-like structure (Fig. 1a), each subunit protruding into a pincer (the respective parts are called the β-protrusion and β-lobe [β-domain 1 and 2] and the β′-clamp). Subunits β and β′ then form three channels.2 The opening between the β/β′ pincers forms the primary channel where nucleic acids bind. The primary channel is separated by the bridge helix (BH; in β′) from the secondary channel, through which nucleoside triphosphates or other substrates access the active site (AS) that is positioned at the junction of the two channels. Next to the BH, the trigger loop (TL, in β′) is found; these two elements participate in the nucleotide addition cycle.

Finally, the RNA exit channel lies on the opposite side of the RNAP core where nascent RNA passes between the base of the β-flap and the β′-lid. In the RNAP elongation complex (Thermus thermophilus, PDB ID 2O5J),1 the downstream DNA (dwDNA) enters the complex through a cleft between the β′-clamp, β′-jaw, and β-lobe; the template strand then reaches the AS around the BH, and the DNA/RNA hybrid is held between β′-rudder, β′-lid, and β-protrusion.

Besides the RNAP subunits that are conserved in all bacteria, some species contain additional subunits, such as δ and ε that are present in Firmicutes.2 Another transcription factor is HelD,9 a protein similar to SF1 helicases that associates with the RNAP core in the model Gram-positive bacterium Bacillus subtilis (Bsu) where it was shown to be involved in transcriptional recycling.11 Bsu HelD binds and hydrolyzes ATP and this is accompanied by conformational changes in the protein as demonstrated by SAXS experiments.12 The absence of HelD from Bsu cells results in a prolonged lag phase during outgrowth of stationary phase cells when diluted into fresh medium.11 Overexpression of HelD then

![Fig. 1 Cryo-EM structures of Msm HelD–RNAP complexes.](image)

**Fig. 1** Cryo-EM structures of Msm HelD–RNAP complexes. a Description of Msm RNAP core (PDB ID 6F6W) subunits and domains; RNAP subunits are color-coded according to the inset legend. b–d Atomic model surface representation of three identified Msm HelD–RNAP complexes: State I – PCh-engaged, State II – PCh-engaged AS-interfering, and State III – PCh-dis-engaged AS-interfering. When fully ordered in State I and II (b, c), the HelD protein (color-coded as in e) forms a crescent-like shape, ends of which protrude to the primary and secondary channels of the RNAP core. The partly ordered HelD protein in State III (d) vacates most of the RNAP primary channel. e Schematic linear representation of the domain structure of the HelD protein. The 1A domain (two shades of yellow) is split in aa sequence into two parts, separated by a large HelD-specific insertion (hues of blue and orange). The nucleotide-binding motifs are marked as vertical thick black lines. As numbering (Msm) is shown below. f–h Three states of HelD as observed in b–d color-coded according to the domain structure (e); secondary structure elements are marked as in Supplementary Fig. 7a.
accelerates spore formation\textsuperscript{13}. However, the structure of HelD, its binding mode to RNAP, and mechanistic details of its function are unknown.

Here, we present structural data for HelD from \textit{Mycobacterium smegmatis} (\textit{Msm}) in complex with the RNAP core and provide insights into its function. We solved the 3D structures of three complexes of \textit{Msm} RNAP and HelD by cryogenic electron microscopy (cryo-EM). The structures represent a so far unknown type of interaction between an RNAP and a protein. The structures suggested the possibility of simultaneous binding of HelD and $\sigma^{\ast}$ to RNAP, and by immunoprecipitation experiments we detected this transitional complex in the cell. Next, we provide biochemical evidence showing that in addition to being able to hydrolyze ATP, HelD can also hydrolyze GTP. Finally, we demonstrate that HelD can both prevent binding of the RNAP core to non-specific DNA and actively remove RNAP from stalled elongation complexes. Together, the results provide the basis for defining the role of HelD in the transcriptional cycle.

**Results**

**Cryo-EM of \textit{Msm} RNAP–HelD complex.** Our long-term attempts to crystallize \textit{Bsu} HelD, RNAP core, or their complex failed; our cryo-EM experiments with the \textit{Bsu} RNAP core were not successful; also, our recent SAXS-based data for the \textit{Bsu} HelD–RNAP complex were not fully conclusive. However, in co-immunoprecipitation experiments with \textit{Msm} RNAP, we identified MSMEG\textunderscore 2174, a potential homolog of \textit{Bsu} HelD (Supplementary Fig. 1). We also solved the X-ray crystal structure of \textit{Bsu} HelD N-terminal domain (CTD), which was then used as a guide for building the model of \textit{Msm} HelD.

We reconstituted a complex of the \textit{Msm} RNAP core and \textit{Msm} HelD from purified recombinant proteins (Supplementary Fig. 2), and froze an isolated homogenous fraction of the complex on cryo-EM grids. We collected multiple preliminary cryo-EM data sets, which allowed us to optimize the cryo-EM conditions for high-resolution three-dimensional (3D) single-particle reconstructions (Supplementary Figs. 3–6). We identified two major 3D classes (State I and State II, Supplementary Fig. 4) at overall resolution $\sim 3.1\AA$ (plus one subclass at $\sim 3.6\AA$), visualizing almost the complete structure of HelD bound to the RNAP core in two conformations (Fig. 1b, c, and Supplementary Movies 1, 2), and one minor class (State III; Supplementary Fig. 4), at $\sim 3.5\AA$, which delineates only two domains of HelD binding to the RNAP core (Fig. 1d and Supplementary Movie 3).

The structures of States I and II share the same overall fold of HelD, with a crescent-like shape (Fig. 1b, c). The main body of the crescent is sitting in between the $\beta$-lobe, the cleft/jaw, and the funnel/secondary channel of the $\beta'$ subunit, burying about 774 and 2608 $\AA^2$ in State I and 1490 and 3623 $\AA^2$ in State II of the binding surface area of $\beta$ and $\beta'$ subunits, respectively\textsuperscript{14}. One end of the crescent protrudes deep into the primary channel, and the other end into the secondary channel of the RNAP core. Indeed, to be able to reach both RNAP channels simultaneously, the HelD protein is markedly elongated, around 200 $\AA$ along the outer edge of the virtual crescent, and the two ends of the HelD protein are separated by $\sim 75\AA$ (State II; Fig. 1c).

The HelD protein itself is divided into six structured domains (Fig. 1e–h), several of which possess unique, so far unknown folds. Interestingly, the 1A domain is composed of two parts (1A-1 and 1A-2) that are separated in the primary amino acid sequence by the intervening HelD-specific domain. According to the position of the HelD domains within the primary channel (PCh) and active site (AS), we name State I: PCh-engaged, State II: PCh-engaged and AS-interfering, and State III: PCh dis-engaged and AS-interfering (Fig. 1b–d).

**The HelD N-terminal domain inserts into the RNAP secondary channel.** The \textit{Msm} N-terminal domain (HelD/1–144) forms an antiparallel $\alpha$-helical coiled-coil (NCC) (HelD/1–69) followed by, and packed against the four-$\beta$-strand globular (NG) domain (HelD/70–144), which contains an additional prominent protruding loop (NG-loop, residues HelD/88–103; Figs. 1e–h and 2a, b). The overall N-terminal domain structure is analogous to the archetypal fold interacting with the secondary channel of RNAP present in transcription factors such as GreA or ppGpp cofactor DksA\textsuperscript{15–17}. Indeed, the HelD N-terminal domain interacts tightly with the secondary channel, burying $\sim 1790\AA^2$ of the interaction surface, contributing largely to the HelD–RNAP interaction. Several specific hydrogen bonds and salt bridges (Supplementary Table 1) are formed between the N-terminal domain and the secondary channel, and particularly the NG-loop specifically recognizes the tip of the coiled-coil (CC) motif of the $\beta'$-funnel (Fig. 2a).

The topology of the \textit{Msm} HelD NCC is conserved in comparison with other secondary channel-interacting transcription factors (Supplementary Fig. 8); however, in contrast to the known structures of such complexes, the \textit{Msm} HelD NCC is shorter and its tip does not reach into the AS (Supplementary Fig. 8). Indeed, a large part of the NCC is extensively packed with the NG-domain into a common hydrophobic core, thereby preventing the NCC to bind further towards the AS. The HelD NCC tip is positioned at the level of the RNAP AS $\beta'$ bridge helix ($\beta'$-BH), $\sim 10$–12 $\AA$ away from Mg$^{2+}$ metal A (MgA) of the AS, and as a result, it constitutes one wall of the secondary channel pore leading to the AS. The pore itself is $\sim 11\AA$ wide (Fig. 2b) and this would still allow nucleoside triphosphate (NTP) passage into the AS. On the other hand, the NCC-domain restricts the conformational freedom and induces folding of the AS trigger loop ($\beta'$-TL, $\beta'$/1009–1028). This would likely interfere with the nucleotide addition cycle.

Another difference with respect to GreA family transcription factors is that the HelD NCC tip does not contain the conserved DXX(E/D)\textsuperscript{18–20} motif (Supplementary Fig. 8), and it is, therefore, unlikely that the \textit{Msm} HelD N-terminal domain possesses a Gre factor-like endonuclease activity.

**The NTPase unit of HelD is positioned in the vicinity of the downstream section of the primary channel.** The presented structure confirms our previous prediction\textsuperscript{12} that HelD, similarly to SF1 helicases, RapA and UvrD, contains a conserved Rossmann fold 1A–2A heterodimer. Domain 1A is formed by two sub-domains 1A-1 and 1A-2 separated in amino acid sequence by the HelD-specific part (Fig. 1e). 1A-1 is connected with the N-terminal domain by the NG-linker (HelD/70–103), which orders only in State I. 1A-2 is then followed by 2A (Figs. 1e–g and 2c).

The 1A domain docks on the $\beta$-lobe where it induces small changes in domain orientation and conformation and it prolongs the wall of the downstream section of the primary channel along the axis of the virtual dwDNA (Fig. 2c). The 1A domain buries an area of 725 $\AA^2$ of the interaction surface of the $\beta$-lobe, and the binding also involves the ordering of the $\beta$-turn $\beta$209–212 and many hydrogen bonds and salt bridges (Supplementary Table 2). In addition, the extension of the 1A domain (HelD/504–521) is clamped in between the prominent $\beta$-turn $\beta$184–187 of the $\beta$-lobe and the tip of the $\beta'$-jaw, further securing the 1A domain in its place (Fig. 2c).

The 1A–2A heterodimer establishes the canonical tertiary structure to form an NTP-binding pocket. Conserved residues of motifs Q, I, II, ~III, IIIa, Va, and VI are then likely involved in NTP binding\textsuperscript{21} (Fig. 2d), while residues typical for DNA binding (in SF1 helicases) are missing. However, the base type specificity
is not obvious from the structural data and, therefore, we measured nucleoside triphosphate hydrolysis activity of the isolated HelD protein. HelD showed strong hydrolysis activity of purine base triphosphates but no activity towards a pyrimidine-containing counterpart (Fig. 2e and Supplementary Fig. 8d). Similarly, the ssDNA-binding motifs negatively charged surface patch in the equivalent areas (Supplementary Fig. 9d, e). Similarly, the ssDNA-binding motifs are not conserved in HelD. Instead, HelD contains proline-rich loops in place of these motifs and a large conserved domain (α5-β2-α3-β3-α4-β4-α5-β5-α6-β6-α7-β7-α8). Next, we measured ATPase and GTPase activities but did not hydrolyze CTP. The apparent negative value of CTP hydrolysis was caused by high background readings. The bars show mean values, the error bars indicate ±SD and the individual symbols represent values from three independent replicates. The data were analyzed and the graphics created with GraphPad Prism 7.02.

**The Msm HelD-specific domain is inserted into the downstream section of the RNAP primary channel.** The HelD-specific insertion domain is composed of the clamp-opening domain (CO-domain, HelD/261–447) and the primary channel loop (PCh-loop, HelD/448–503) (Figs. 1e and 3b–e). The CO-domain is an extended, mostly α-helical, and so far undescribed fold with no structural homologs (Supplementary Fig. 7b). On one side, the CO-domain packs against the 1A domain helix α19 and β-turn HelD/561–564. Additionally, the CO-1A interaction is stabilized by the CO-linker (HelD/259–275), which connects the two domains. In State I, the other side of the CO-domain, the CO-tip, butts against the three-stranded sheet of the β′ non-conserved domain (β′-NCD) and an α-helix (β′/122–133) of the β′-clamp just preceding it (Fig. 3a, b, d). The only significant ordered part of the PCh-loop in State I, the protruding helix α16 (HelD/451–468), is erected against the β′ three-stranded sheet (β′/1164–1210) and the α16 tip locks behind the helix-turn-helix motif β′/271–304 by HelD/Tyr466. Altogether, the α16
interaction with the β′-clamp might be helping the CO-domain insertion into the primary channel. In State II, the CO-domain fold alters and the PCh-loop completely refolds. The CO-domain tip shifts towards the β′ clamp coiled-coil domain (β′-CC) domain and reaches the peptide β′/387–389 of the rudder (Fig. 3c, e). The PCh-loop protruding helix α16 refolds (α16 register slightly shifts towards the C-terminus of HelD) and dis-engages with the β′ three-stranded sheet (β′/1164–1210), and the whole PCh-loop orders towards the AS (see next section). Correspondingly, the two insertion modes of the CO-domain and PCh-loop into the primary channel force the β′-clamp domain to swing out into two distinct positions (see details below).

The HelD PCh-loop is able to fold into the RNAP active site. In the cryo-EM map of the AS-interfering State II, high-resolution density is present for the entire register of the PCh-loop, which is
folded in the AS cavity of RNAP (Fig. 3c, f, g and Supplementary Fig. 5c). The folding of the PCh-loop in between the walls of the AS chamber is also compatible with the regular open form of the RNAP core as observed in State III.

In comparison to State I in State II the protruding helix α16 refolds, the helix register shifts to residues 455–472, and together with a newly folded helix α17 (HelD/495–500) they tightly pack with the second half of the β'-BH (Fig. 3g). In detail, BH β'/Arg874 and Arg875 sandwich α16 HelD/Tyr466 and, cooperatively, BH β'/Tyr871 stacks on HelD/Phes502 and is inserted into a hydrophobic pocket formed by HelD/Tyr466, Ala467, Val470, and Leu498. The rest of the PCh-loop (HelD/473–494) specifically wedges into the AS cavity (Supplementary Table 3), towards the AS aspartate triad and MgA. Notably, there are four acidic residues (482–DDED-485) at the very tip of the PCh-loop and the HelD/481–483 peptide rods along the AS β-strap β'/537–544, such that HelD/Asp483 is in contact with MgA and HelD/Asp482 in its near proximity (Fig. 3f and Supplementary Fig. 5c). HelD/Asp482 interacts with β'/Arg500, HelD/Asp484 stabilizes the loop in the active site by interaction with β'/His1026, and HelD/Asp485 contributes to the AS-interfering loop stability by a salt bridge with the side chain of HelD/Arg477. Two other motifs support the formation of the PCh-loop structure in the RNAP AS—a small hydrophobic core formed by the HelD/Val475, Leu480, and Leu488 side chains and an intra-chain ion-pair HelD/Arg477–Asp491, with HelD/Arg477 leaning against β'/Pro483.

As a result of the PCh-loop folding into the primary channel and HelD NCC folding in the secondary channel, the NCC tip opens the secondary channel and its influence on the rest of the complex remains very similar in all the States. This interaction thus might be the initial one through which HelD starts its association with RNAP. Furthermore, this interaction seems sufficient to alter the position of the β'-jaw/cleft and β'-lobe (Supplementary Fig. 10g) which may weaken interaction with dwDNA, reminiscent of TraR (a distant DksA homolog) binding to E. coli RNAP25.

The main change between the States is the interplay between the refolding of the PCh-loop and the CO-domain position in the primary channel. In State III, solely the PCh-loop’s tight contact with the AS stabilizes a very open form of RNAP (Supplementary Fig. 10a, b, f), ~33 Å at the narrowest point of the primary channel (measured by the distance of the Cα atoms of β'/Lys273 and β'/Lys123), comparable to the structures of two previously identified conformations of very open forms of Msm RNAP core and holoenzyme, termed Core2 and Holo2 (32.2 and 33.6 Å, respectively)11. In State I, the PCh-loop’s interaction with β'/helix-turn-helix and three-stranded sheet, and the CO-domain insertion into the primary channel make the opening of the RNAP clamp (~35 Å; Supplementary Fig. 10a, b) slightly wider than the already widely open forms of the Lipiarmycin–26 (PDB ID 6FBV) and Fidaxomicin-locked27 (PDB ID 6C06) RNAPs (34.2 and 33.6 Å, respectively)11. In State II (Supplementary Fig. 10e), while the CO-domain still inserted, the PCh-loop abolishes the β' contact and folds in the AS instead, and this forces the β'-clamp (β'/1–406) to rotate with respect to the remaining parts of the complex so that the β'-NCD CC tip opens further away from the juxtaposed β'-lobe but at the same time the β'-rudder, β'-CC, and adjacent secondary elements move about 11 Å closer to the tip of the HelD CO-domain. The RNAP clamp is, therefore, splayed by 45 Å (Supplementary Fig. 10b). This clamp opening together with the tight interaction of the PCh-loop with the AS is not compatible with nucleic acid binding.
The next major differences are the β-lobe and CO-domain adjustments upon change of the 1A–2A heterodimer (Supplementary Fig. 10e). The mutual orientation of 1A and 2A domains between States I and II is almost preserved, although with much poorer density for 2A in State II. This most likely stems from the more pronounced mobility of 2A, possibly linked with the lack of stabilization by the unfolded NG-linker in State II. The 2A relaxation allows movement of 1A in respect to the N-terminal domain (~3° difference measured by HeLD a1 and a5) and a concomitant shift of both the β-lobe and CO-domain (Supplementary Fig. 10e). In detail, this global change is accompanied by a shift and changes in the secondary structure of HeLD/230–252 within the 1A domain (largest shift about 9.3 Å for Val245). Helix a6 is extended and helix a7 is formed in State II (Supplementary Fig. 7a) and 1A-extension shifted. State I interactions between a6 and the NTPase site, and a6 and the NG-linker that are NTP-binding prohibitive, are broken in State II and the NTPase site of HeLD becomes wide open (NTP-binding permissive; Supplementary Fig. 10h). Although this change makes the NTPase site accessible for NTPs, additional conformational changes are still required for NTP accommodation.

Finally, HeLD binding in States I and II also leads to the opening of the RNA exit channel between the β-flap and β'-lid and β'-Zn-finger by about 15 and 21 Å, respectively (Supplementary Fig. 10c, d). State III keeps the channel still rather open by about 12 Å. This is expected to contribute to RNA release.

**HeLD clears the RNAP primary channel.** The position of the HeLD CO-domain in the primary channel of RNAP suggests that HeLD may prevent non-specific interactions between the RNAP core and DNA. To test this, we performed an electrophoretic mobility shift assay (EMSA) with RNAP and a fragment of mycobacterial DNA in the presence/absence of HeLD. Figure 4a–c shows that HeLD significantly abolishes the non-specific binding of the RNAP core to DNA.

Moreover, we speculated that HeLD might not only prevent DNA binding but also actively disassemble stalled ECs. Stalled ECs (due to, e.g., damaged DNA) are obstacles for both the coupled transcription–translation machinery and also for replication, with potentially deleterious consequences if not removed. To test the ability of HeLD to rescue stalled RNAP, we assembled ECs with the RNAP core on a DNA–RNA scaffold and challenged them with HeLD in the presence/absence of NTPs (Fig. 4d). HeLD then, relative to mock treatment, was able to disassemble stalled ECs (Fig. 4e). This process, interestingly, appeared to be independent of ATP or GTP.

HeLD, σA, and RbpA can simultaneously bind RNAP core. Analysis of States I–III suggested the possibility of simultaneous binding of HeLD, σA, and RbpA to RNAP. Modeling of hypothetical complexes of RNAP–HeLD with σA and RbpA then confirmed that relatively small changes in conformations of these proteins could allow their simultaneous binding to RNAP in States I–III (Supplementary Fig. 11 and “Discussion”). Therefore, we tested experimentally whether the HeLD–RNAP complex is compatible with the presence of other factors. Indeed, immunoprecipitation (IP) and western blot experiments with FLAG-tagged *Msm* RNAP revealed the presence of HeLD and σA (Fig. 4f, g); FLAG-tagged *Msm* σA pulled down the RNAP core and HeLD; FLAG-tagged HeLD pulled down the RNAP core and σA. These results suggested but not proved that HeLD, σA, and RNAP are together in one complex. Alternatively, HeLD and σA could bind each other independently of RNAP. To decide between the two possibilities, we first pulled down FLAG-tagged HeLD and associated proteins, and from this mixture, we subsequently pulled down σA (with an antibody against σA) and associated proteins. Supplementary Fig. 12 shows the presence of HeLD and RNAP in the second pull-down, demonstrating that all these proteins (RNAP, σA, HeLD) can coexist in one complex. Additionally, RbpA, albeit in low amounts, was also present in the HeLD-immunoprecipitated complex and RbpA–FLAG pulled down RNAP with σA and HeLD (Supplementary Fig. 13). We then confirmed the interactions between the RNAP core, σA, RbpA, and HeLD by in vitro EMSA (Fig. 4h).

**Discussion**

This study describes a structurally unique complex between *Msm* RNAP and the HeLD protein, defines its DNA-cleaving activity, and outlines its role in transcription.

**Comparison of *M. smegmatis* and *B. subtilis* HeLD.** Previous biochemical studies used HeLD from *B. subtilis*, which is only 21% identical with the *Msm* homolog. Selected sequence homologs of *Msm* HeLD are shown in Supplementary Fig. 14, revealing two main differences between *Msm* (Actinobacteria) and *Bsu* (Firmicutes). The first marked difference is the absence of ~30 aa from the N-terminal NCC-domain region in *Msm* HeLD. This is consistent with the *Bsu* HeLD NCC-domain protruding much deeper into the RNAP secondary channel and even overlapping with the AS31,32. The other difference is in the HeLD-specific region where *Bsu* HeLD completely lacks the PCh-loop. On the other hand, the organization of the 1A-1 and 1A-2 split followed by the complete 2A domain is maintained (Fig. 1e, f, g).

Interestingly, *Msm* HeLD, σA, and RbpA can co-occur on RNAP (Fig. 4h and Supplementary Figs. 11–13) and we infer that the RNAP–σA–RbpA–HeLD complex thus likely represents one of the possible transitional states in the transcriptional cycle. This differs from *Bsu* where simultaneous HeLD and σA binding has not been detected11. Regardless of the exact mutual positions of σA, RbpA, and HeLD, RNAP must subsequently assume a conformation that is compatible with promoter DNA binding and transcription initiation.

**Model of the HeLD role in transcription.** Based on the structural and functional data we propose a role for *Msm* HeLD in transcription (a model is shown in Fig. 5). We envisage that upon transcription termination when RNAP fails to dissociate from nucleic acids33, or in the event of stalled elongation, *Msm* HeLD first interacts with RNAP by its N-terminal domain, likely competing for binding to the secondary channel with GreA-like factors. This initial HeLD binding induces changes in β-lobe and β'-jaw/cleft (Supplementary Fig. 10g), possibly leading to destabilization of dwDNA in the primary channel. The trigger loop is conformationally locked. Subsequently, the CO-domain and PCh-loop approach the primary channel. The PCh-loop, which is probably flexible in the RNAP-unbound state, folds partially upon binding RNAP (captured in State I), and then it penetrates deep into the primary channel, fully folds, and binds to the AS (captured in States II and III). The CO-domain interactions with β'-clamp then secure the primary channel wide open (Supplementary Fig. 10a, b). At the same time, the RNA exit channel dilates (Supplementary Fig. 10c, d). All these processes lead to the release of any contents of the AS (compare states within Supplementary Fig. 10a).

We note that neither HeLD loading onto RNAP nor RNAP clamp opening nor EC disassembly is dependent on NTP hydrolysis. Energy from NTP hydrolysis is probably required to release HeLD from its tight contact with RNAP. Free energy corresponding to ATP hydrolysis under physiological conditions in cells is around ~50 kJ/mol34. This is comparable to the
estimation of desolvation energy of the HelD–RNAP core interaction of $-33.5\text{ kJ/mol (A/G)}$ for State I and $-57\text{ kJ/mol}$ for State II. However, States I and II are not fully comparable with canonical NTP binding in the HelD NTPase unit. It remains to be answered which structural changes are required to actually enable NTP binding and hydrolysis.

To summarize, HelD clears RNAP of nucleic acids; this likely happens in non-functional (e.g., stalled) transcription complexes or post-termination. This may contribute to the smooth transcription inhibition, as seen, e.g., in the action of Fidaxomicin towards M. tuberculosis RNAP in complex with RbpA.$^{27}$

**Methods**

**Bacterial strains, plasmids, and oligonucleotides.** Bacterial strains and plasmids are listed in Supplementary Table 4. DNA oligonucleotides are listed in Supplementary Table 5.

**Strain construction—$\sigma^A$ and RbpA.** $\sigma^A$ (MSMEG_2758) and RbpA (MSMEG_3858) genes were amplified from genomic DNA by PCR with Phusion High-Fidelity DNA Polymerase (NEB) with primers #1155 + #1156 ($\sigma^A$) and #1182 + #1183 (RbpA) and Ms1$^{36}$, which keep RNAP in an inactive state under growth- unfavorable conditions. This stored RNAP then accelerates the restart of gene expression when conditions improve.

Finally, the RNAP-inactivating ability of HelD might be utilized in the development of specific antibacterial compounds that would stabilize the non-productive HelD–RNAP complex, shifting the equilibrium of RNAP states towards effective transcription inhibition, as seen, e.g., in the action of Fidaxomicin towards M. tuberculosis RNAP in complex with RbpA.$^{27}$
700084/mc² (155) with cleavage site for TEV protease placed at the 5' end. This synthesized gene was cloned into the Champion vector (Thermo Scientific) and proteins were eluted with a linear gradient of imidazole to the final concentration of 400 mM over 20 column volumes. The Msm RNAP core elution fractions were pooled and dialyzed to 20 mM Tris–HCl pH 8 (4 °C), 1 mM NaCl, 5% (v/v) glycerol and 4 mM dithiothreitol (DTT) for 20 h. The protein was further polished on AK 26/70 Superose 6 pg column (GE Healthcare) equilibrated in 20 mM Tris–HCl pH 8 (4 °C), 300 mM NaCl, 5% (v/v) glycerol and 4 mM DTT. The Msm RNAP core final fractions were eluted at 6 μM concentration, aliquoted, flash-frozen in liquid nitrogen, and then stored at −80°C.

**Strain construction—HeID-FLAG, α²-FLAG, and RbpA-FLAG.** The genes coding for the HeID-FLAG, α²-FLAG, and RbpA-FLAG proteins were amplified by PCR using Q5 High-Fidelity DNA Polymerase (NEB) with primers #3130 + #3131 (HeID), #2339 + #2340 (α²) and #2894 + #3093 (RbpA) and Msm chromosomal DNA as the template. The C-terminal xFLAG-tag (DYKDDDDK) were encoded within the reverse PCR primers for all genes. Subsequently, the genes were inserted into integrative plasmid pTetInt37 via restriction sites. The resulting plasmids were verified by sequencing. The resulting plasmids were transformed into Msm mc² 155 (wt, LK865) cells by electroporation resulting in strains LK2590 (HeID-FLAG), LK2073 (α²-FLAG), and LK2541 (RbpA-FLAG).

**Growth conditions.** Msm strains—mc² 155 (wt, LK865), α²-FLAG (LK2373), RNAP-FLAG (LK1468), HeID-FLAG (LK2590), and RbpA-FLAG (LK2541) were grown at 37 °C in Middlebrook 7H9 medium with 0.2% glycerol and 0.05% Tween 80 and harvested in exponential phase (OD₆₀₀ ~ 0.5; 6 h of cultivation) or early stationary phase (OD₆₀₀ ~ 2.5–3.0, 24 h of cultivation) unless stated otherwise. When required, media were supplemented with kanamycin (20 μg/ml). Expression of HeID-FLAG in exponential phase was induced by anhydrotetracycline (1 ng/ml) at 3 h of cultivation. The cells were then grown for an additional 3 h. Expressions of α²-FLAG, RbpA-FLAG, and HeID-FLAG in the stationary phase were induced by anhydrotetracycline (10 ng/ml) at 8 h of cultivation. The cells were then cultivated for an additional 16 h.

**Msm RNAP core purification for cryo-EM.** Eco strain Lemo 21 (DE3) was transformed with pET302/NT-His (cmlr and ampr) plasmid derivative encoding the Msm HeID protein fusion with N-terminal 6xHis tag under the control of the T7 promoter. Expression cultures were incubated at 37 °C and shaken at 230 rpm until OD₆₀₀ ~ 0.8; expression was induced with 500 μM IPTG at 17 °C for 16 h. Cells were lysed using sonication by Sonic Disembrator Model 705 (Fisher Scientific) in a lysis buffer containing 50 mM Tris–HCl pH 7.5 (4 °C), 400 mM NaCl, 30 mM imidazole, 0.2% Tween20, 2 mM β-mercaptoethanol, EDTA-free protease inhibitor cocktail (Roche), RNase A (Sigma), DNase I (Sigma), and Lysozyme (Sigma). Clarified lysate was loaded onto a HiTrap FF Crude column (GE Healthcare) and proteins were eluted with a linear gradient of imidazole to the final concentration of 400 mM over 20 column volumes. Fractions containing HeID protein were pooled and dialyzed for 20 h against the dialysis buffer containing 20 mM Tris–HCl, pH 7.5 (4 °C), 500 mM NaCl, 1 mM DTT together with TEV protease at a TEV protease:HeID ratio 1:20.

The protein was then concentrated to ~15 A₂₆₀ units and further purified using size-exclusion chromatography using a Superdex 75 column (GE Healthcare) equilibrated in 20 mM Tris–HCl, pH 7.5 (4 °C), 200 mM NaCl and 1 mM DTT. The HeID protein was eluted at ~160 μM concentration, aliquoted, flash-frozen in liquid nitrogen, and then stored at −80°C.

**In vitro HeID-RNAP complex reconstitution for cryo-EM.** To assemble the HeID–RNAP complex, the individual proteins were mixed at a molar ratio of 3:1. The in vitro reconstructions were carried out at 4 °C, and the reconstitution mixture was incubated for 15 min. 50 μl of the reconstitution mixture was injected onto a Superose 6 Increase 3.2/300 column (GE Healthcare) equilibrated in 20 mM Tris–HCl, pH 7.8 (4 °C), 150 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. 50-μl fractions were collected and the protein was eluted at ~1 μM concentration.

**Electron microscopy.** Complexes were diluted to ~850 nM and aliquots of 3 μl were applied to Quantifoil R1 2/1.3 or R2/2 Au 300 mesh grids, immediately blotted for 2 s, and plunged into liquid ethane using an FEI Vitrobot IV (4 °C, 100% humidity).
The grids were loaded into an FEI Titan Krios electron microscope at the European Synchrotron Radiation Facility (ESRF) (beamline CM01, ESRF, Grenoble, France). To the HELC (Masaryk University, Brno), operated at an accelerating voltage of 300 keV and equipped with a post-GIF K2 Summit direct electron camera (Gatan) operated in counting mode. Cryo-EM data was acquired using EPU software (FEI) at a nominal magnification of ×165,000, with a pixel size of 0.8311 and 0.840 Å per pixel. Movies of a total fluence of ~40–80 electrons per Å² were acquired at ~1 e⁻/Å² per frame. A total number of 15,177 movies were acquired at a defocus range from ~0.7 to ~3.5 μm (Supplementary Table 6).

**Cryo-EM image processing.** All movie frames from three data sets were aligned and dose-weighted using the MotionCor2 program (Supplementary Fig. 3a) and then used for contrast transfer function parameter calculation with Gctf48. Initially, particles were selected without a template by Goutam (provided by Dr. Kai Zhang, http://www.mrc-lmb.cam.ac.uk/kaihang) from a small portion of the data set (~1%). The initial small dataset was then used for a three-dimensional classification using RELION 3.0. Eighty representative classes of different views were selected from the two-dimensional averages and used as a reference for automatic particle picking for the dataset 1 by RELION. WARP46 was used for particle picking for data sets II and III. The resulting particles were iteratively subjected to two rounds of 2D-classification (Supplementary Fig. 3b) at 3x and 2x binned pixel size. Particles in classes with poor structural features were removed. Particles from data sets I and II were globally refined to estimate the pixel size matching41 and particles from dataset II were estimated to match the common pixel size 0.8311 Å per pixel. Particles from all data sets were pooled (~1,500,000), 2x binned, and particles from three-dimensional classifications with image alignment (Supplementary Fig. 4). The first round of 3D classification was restricted to ten classes and performed using Msm RNAP core (PDB ID 6fsw) as a 60 Å low-pass filtered initial model. The directional resolution anisotropy (Supplementary Fig. 5a) was estimated by LocScale44. The directional resolution anisotropy (Supplementary Table 6) and the model resolution was estimated at the 0.5 FSC cut off. Structures were analyzed and Figures were prepared using the following software: Structures were analyzed and Figures were prepared using the following software:

**X-ray crystal structure determination of the Bsu HelD C-terminal domain.** The DNA sequence encoding the C-terminal domain of HelD (from residue 608 to 774) was amplified by PCR and cloned into pET15b vector by NdeI and BamHI restriction enzymes to generate N-terminal His6-tagged protein. A bacterial culture containing BL21(DE3) RIPL codon-plus cells transformed with a pET15b–HelD-CTD vector was grown at 37°C in LB medium supplemented with 100 μg/ml ampicillin, protein expression was induced with 0.5 μM IPTG at OD⁰₆₀ = 0.5, and incubated for additional 3 h to allow protein expression. Cells were harvested by centrifugation and the sonications in ice (pH 8.0 at 4°C, 200 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol, 2 mM p-Nethylmethylenesulfonyl fluoride, PMSF). The lysate was clarified by centrifugation and HelD-CTD was purified by Ni-NTA, Q-Sepharose, and Heparin-column chromatography. Fractions containing HelD-CTD were concentrated using VivaSpin concentrators until 10 mg/ml in crystallization buffer (10 mM Tris–HCl, pH 8 at 4°C, 50 mM NaCl, 1% glycerol, 0.1 mM EDTA, 1 mM DTT).

Crystalization condition of HelD-CTD was screened by using the JCSG+ screen (Molecular Dimensions) and crystals were obtained in crystallization solution (0.1 M Na/K phosphate, pH 6.2, 0.2 M NaCl, 50% PEG200) at 22°C. X-ray crystallographic data were collected using the Penn State X-ray Crystallography Facility and the data were processed with HKL200056. For sulfur single-wavelength anomalous dispersion phasing, 10 S atom positions were identified and the initial and final phase and density-modified maps were calculated by AutoSol followed by automatic model building by AutoBuild in the program Emsl.42 Here refinement by Phenix and model building using Coot48 improved the map and final. Finally, water molecules were added to the model. The data statistics and X-ray structure parameters are included in Supplementary Table 7.

**Protein purification for biochemical assays—Msm RNAP core.** A strain of E. coli containing plasmid with subunits of the RNAP core (Lk1853) was grown to an OD600 ~0.5. Expression of RNAP was induced with 500 μM IPTG for 4 h at room temperature. Cells were harvested by centrifugation, washed, resuspended in P buffer (300 mM NaCl, 50 mM Na2HPO4, 5% glycerol, 3 mM β-mercaptoethanol), and disrupted by sonication. Cell debris was removed by centrifugation and supernatant was mixed with 1 ml Ni-NTA Agarose (Qagen) and incubated for 90 min at 4°C with gentle shaking. Ni-NTA Agarose with bound RNAP was loaded on a Poly-Prep® Chromatography Column (Bio-RAD), washed with P buffer and, subsequently, washed with P buffer with 30 mM imidazole. The proteins were eluted with P buffer containing 400 mM imidazole and fractions containing RNAP were pooled and dialyzed against storage buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 50% glycerol, 3 mM β-mercaptoethanol). The RNAP protein was stored at ~20°C.

**Protein purification for biochemical assays—Msm RbpA.** Expression strain of E. coli containing plasmid with gene of o⁶ (Lk1740) was grown at 37°C until OD600 reached ~0.5; expression of o⁶ was induced with 300 μM IPTG at room temperature for 3 h. Isolation of o⁶ was done in the same way as for RNAP purification with the exception of 50 mM imidazole added to the P buffer before resuspending the cells. Instead of the purification in a column, batch purification and centrifugation were used to separate the matrix and the eluate.

**Protein purification for biochemical assays—Msm HelD.** Msm HelD was prepared as described previously, in the paragraph about the purification of proteins for cryo-EM experiments. The purity of all purified proteins was checked by SDS-PAGE gel.

**Msm HelD ATP, GTP, and GTP hydrolysis assay.** Hydrolysis of ATP, GTP, and CTP (Sigma-Aldrich) by Msm HelD was measured in a total volume of 50 μl of reaction mixture which contained 10 mM substrate, 10 μg of Msm HelD, and 0.4 mM MgCl₂ (Phosphate buffered 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂). Incubation was carried out at 37°C for 30 min. The amount of released phosphate was analyzed spectrophotometrically at A = 850 nm according to a modified molybdenum blue method57 using a microplate reader Clariostar (MBG LAB-TECH, Ortenberg, Germany). Briefly, the reaction was stopped by adding 62 μl of reagent A (0.1 M m-scobic acid, 0.3 M Cl₂(COÖH)). After thorough mixing, 12.5 μl of reagent B (10 mM (NH₄)₂Mo₇O₂₄) and 32 μl of reagent C (0.1 M sodium citrate, 0.2% Na₂MoO₄, 10% acetic acid) was added. All enzymatic reactions were performed in triplicates with separate background readings for each condition.

**DNA–protein interaction analysis in vitro.** DNA–protein interactions were analyzed on 4–16% Bis-Tris native gels (ThermoFisher Scientific, cat. no. BN0102 BOX) by Electrophoretic Mobility Shift Assay (EMSA). The DNA fragment was amplified by Expand High Fidelity PCR System (Roche, cat. no. 41003) in 1×TBS in 1×TBS buffer (50 mM Tris–HCl, pH 8.0; 5 mM MgCl₂; 100 μM DTT; 50 mM KCl; 50 μg/ml BSA) that contained 0.1% Triton X-100. Incubation was carried out at 37°C for 30 min. Following thorough mixing, 12.5 μl of [32P]-labeled DNA and 12.5 μl of reagent C (0.1 M sodium citrate, 0.2% Na₂MoO₄, 10% acetic acid) was added. All enzymatic reactions were performed in triplicates with separate background readings for each condition. The resulting 304-bp-long PCR fragment was excised and purified from agarose gel. Binding reactions were performed in 1×TBS buffer (50 mM Tris–HCl, pH 8.0; 5 mM MgCl₂; 100 μM DTT; 50 mM KCl; 50 μg/ml BSA) that contained 0.1% Triton X-100. Incubation was carried out at 37°C for 30 min. Following thorough mixing, 12.5 μl of [32P]-labeled DNA and 12.5 μl of reagent C (0.1 M sodium citrate, 0.2% Na₂MoO₄, 10% acetic acid) was added. All enzymatic reactions were performed in triplicates with separate background readings for each condition.
was subsequently stained with Simply Blue (Invitrogen, cat. no. LC6060) for protein visualization.

**Protein-protein interaction analysis in vitro.** Protein–protein interactions were analyzed on 7% Tris-acetate native gels (ThermoFisher Scientific, cat. no. EA0355BOX) by EMSA. The binding reaction was done in 20 μl of 1×STB buffer containing RNAP (25 pmol), HelD (125 pmol), αβ (125 pmol), and Rpβ(125 pmol)—protein combinations in reactions are specified in the Fig. 4 legend. First, RNAP was reconstituted with/without HelD (at 37 °C, 45 min). Then Rpβα and/or αβ were added, followed by additional incubation at 37 °C for 45 min. 40 μl of Native Tris-Glycine buffer (Invitrogen, cat. no. LC2673) was added, and 20 μl of the mixture was then loaded on a native gel. Electrophoresis was run in a cold room (4 °C). Subsequently, for protein visualization, the gels were stained with Simply Blue. The identity of proteins in each band was determined by MALDI mass spectrometric identification.

**Disassembly of elongation complexes.** Elongation complexes (ECs), containing a transcription bubble, were assembled with the Msm RNAP core, based on a previously described assay.58 Briefly, DNA and RNA oligonucleotides were purchased and are the same as in Table E7 in ref.7. The RNA (LK-pRNA) was monophosphorylated at the 5′ end by the manufacturer. A 2-fold molar excess of RNA was mixed with template DNA (LK632) in water and annealed in a cycler (45 °C for 2 min, 42–27 °C; the temperature was decreasing by 3 °C every 2 min, 25 °C for 10 min). RNAP (32 pmol per sample) was incubated with 4 pmol of the annealed hybrid in 10 μl of reaction buffer (40 mM Tris–HCl pH 8.0, 10 mM MgCl2, 1 mM DTT) for 15 min at room temperature with gentle shaking. 8 pmol of non-template DNA (LK631) containing RNAP (25 pmol), HelD (125 pmol), αβ (125 pmol), and Rpβ(125 pmol) were added, followed by additional incubation at 37 °C for 45 min. Then Rpβα and/or αβ were added, followed by additional incubation at 37 °C for 45 min. 40 μl of Native Tris-Glycine buffer (Invitrogen, cat. no. LC2673) was added, and 20 μl of the mixture was then loaded on a native gel. Electrophoresis was run in a cold room (4 °C). Subsequently, for protein visualization, the gels were stained with Simply Blue. The identity of proteins in each band was determined by MALDI mass spectrometric identification.

**Trypsin digestion and MALDI mass spectrometric identification.** Simply Blue-stained proteins were cut out from gels, chopped into small pieces, and digested using 50 μM 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile (MeCN). The gel pieces were then washed with water, reduced in size by dehydration in MeCN, and partly dried in a SpeedVac concentrator. The proteins were digested overnight at 37 °C using sequencing grade trypsin (100 ng; Promega) in a buffer containing 25 mM 4-ethylmorpholine acetate and 5% MeCN. The resulting peptides were extracted with 40% MeCN/0.1% TFA (trifluoroacetic acid). For MALDI MS analysis, 0.5 μl of each peptide mixture was deposited on the MALDI plate, air-dried at room temperature, and overlaid with 0.5 μl of the matrix solution (a-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA, 5 mg/ml, Sigma). Peptide mass maps of proteins were generated using a Finnigan Speed MALDI-TOF instrument (Bruker Daltonics, Billerica, USA) in a mass range of 700–4000 Da and calibrated externally using a PepMix II standard (Bruker Daltonics). For protein identification, MS spectra were searched against UniProtKB database of Msm proteins using the in-house MASCOT v.2.6 search engine with the following settings: peptide tolerance of 20 ppm, missed cleavage site set to one, and variable oxidation of methionine. The spectra of proteins in Fig. 4f were acquired on a 15T Solarix XR FT-ICR mass spectrometer (Bruker Daltonics) in a mass range of 500–6000 Da and calibrated internally using peptide masses of Msm RpBo and RpCo proteins. The peak lists generated using DataAnalysis 5.0 program were searched against UniProtKB database of Msm proteins using the in-house MASCOT engine with the following settings: peptide tolerance of 3 ppm, missed cleavage site set to two variable, and oxidation of methionine.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data are available from the corresponding authors. Coordinates and structure factors or maps have been deposited in the wwPDB or EMDB: Bus HelD C-terminal domain (X-ray) PDB ID 6VXV, Msm HelD–RNAP complex State I (cryo-EM) EMD-10996, PDB ID 6YXY, Msm HelD–RNAP complex State II (cryo-EM) EMD-11004, PDB ID 6YYS, Msm HelD–RNAP complex State III (cryo-EM) EMD-11026, PDB ID 6Z11. Source data are provided with this paper.

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