The δ subunit and NTPase HelD institute a two-pronged mechanism for RNA polymerase recycling

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Cellular RNA polymerases (RNAPs) can become trapped on DNA or RNA, threatening genome stability and limiting free enzyme pools, but how RNAP recycling into active states is achieved remains elusive. In Bacillus subtilis, the RNAP δ subunit and NTPase HelD have been implicated in RNAP recycling. We structurally analyzed Bacillus subtilis RNAP-δ-HelD complexes. HelD has two long arms: a Gre cleavage factor-like coiled-coil inserts deep into the RNAP secondary channel, dismantling the active site and displacing RNA, while a unique helical protrusion inserts into the main channel, prying the β and β′ subunits apart and, aided by δ, dislodging DNA. RNAP is recycled when, after releasing trapped nucleic acids, HelD dissociates from the enzyme in an ATP-dependent manner. HelD abundance during slow growth and a dimeric (RNAP-δ-HelD)2 structure that resembles hibernating eukaryotic RNAP I suggest that HelD might also modulate active enzyme pools in response to cellular cues.
Cellular RNA polymerases (RNAPs) are viewed as well-tuned engines that promptly re-initiate a new round of transcription after termination. For example, bacterial RNAPs minimally comprise an αβω subunit catalytic core, which forms a holoenzyme with one of several σ factors to initiate transcription at a promoter. After promoter escape, elongation factors replace σ, and the ensuing elongation complex (EC) synthesizes RNA until a termination signal is reached. At a terminator, bacterial EC is abruptly destabilized either by an oligo-U-tailed G/C-rich RNA hairpin or by the RNA translocase/helicase ρ. However, RNAP can linger on DNA after RNA release, road-blocking replisomes to trigger double-stranded DNA breaks and giving rise to aberrant antisense transcripts. RNAP can also form binary complexes with RNA, either through de novo association with stable RNAs, such as tRNAs and 6S RNA, or in the course of hairpin-induced termination. While some RNA binary complexes serve as RNAP storage depots and can be reactivated when nutrients become available, others may sequester unproductive RNAP.

Post-termination binary complexes have to be dismantled to recycle RNAP, and ordered recycling is considered an integral phase of the duty cycle of many molecular machines, such as ribosomes. By contrast, recycling has so far not garnered similar attention in bacterial transcription. While several accessory proteins could facilitate RNAP detachment from nucleic acids, including σ, transcription repair coupling factor Mfd, ρ, and the NTPase RapA, they release stalled RNAP under specific circumstances rather than act as genuine recycling factors.

RNAPs from some Gram-positive bacteria, including Bacillus subtilis, contain additional small nonessential subunits, and Δω is present in B. subtilis at an equal or higher concentration than standard core subunits, and its expression increases during the transition to the stationary phase, but Δω deletion does not prevent sporulation. Cells lacking the proE gene, encoding δ, have altered morphology and exhibit an extended lag phase and defects in adaptation to changes in growth conditions sensed by initiating NTPs. While a ΔρσE strain has only mild phenotypes, it is not able to compete with the wild type (WT) strain and is required for virulence in Streptococci. δ destabilizes RNAP interactions with promoter DNA, inhibiting initiation at promoters that form unstable open complexes. Consequently, δ suppresses initiation from weak or cryptic promoters, and deletion of proE leads to expression of many otherwise silenced genes in Streptococci. Notably, δ also promotes RNAP recycling by displacing σ from holoenzyme and RNA or DNA from binary complexes. Presently, it is unclear how δ elicits these effects. Likewise, the function of ε remains enigmatic.

HelD, a putative superfamily I nucleic acid-dependent NTPase found in Gram-positive bacteria, is related to Escherichia coli UvrD and Rep helicases and has been implicated in DNA repair and recombination. B. subtilis HelD and RNAP directly interact and are present at comparable levels during sporulation. Together with δ, HelD enhances RNAP cycling, and both proteins are required for adaption to environmental changes.

Based on the above, we hypothesized that HelD is a general recycling factor that acts in collaboration with δ and set out to elucidate its mechanism of action. Using single-particle cryogenic electron microscopy (cryoEM) and cross-linking/mass spectrometry (CLMS), we show that HelD, supported by δ, inserts long prongs into RNAP’s main and secondary channels, competing with bound nucleic acids and prying RNAP open to allow nucleic acid escape. Release assays further support HelD/δ collaboration in RNAP recycling. ATP facilitates HelD detachment and completes RNAP recovery. We also observe RNAP dimerization in the presence of δ and HelD, hinting at a possible role of HelD in RNAP hibernation.

Results

Structural analysis of RNAP-δ-HelD complexes. RNAP fractions enriched from stationary phase B. subtilis cells contained α, β, δ, ε, and ω subunits, with sub-stoichiometric amounts of HelD, PrI A, σδ and σω (Supplementary Fig. 1a). RNAP variants lacking HelD (RNAPδHelD) or lacking δ and HelD (RNAPΔδHelD) were purified from B. subtilis ΔεlD and ΔεlDΔρσE strains, respectively (Supplementary Table 1); RNAPΔδHelD contained δ and ε, yet showed a marked loss of ω (Supplementary Fig. 1b).

We assembled an RNAP-δ-HelD complex by supplementing stationary phase RNAP with δ, HelD, and a DNA/RNA scaffold with an artificial transcription bubble (Supplementary Table 1), followed by size exclusion chromatography (SEC). RNAP bound HelD but not the nucleic acid scaffold, and ω was again underrepresented in the RNAP-δ-HelD fractions (Supplementary Fig. 1c). CryoEM data were collected after vitrifying purified complexes without crosslinking in the presence of detergent to overcome preferred particle orientations (Supplementary Fig. 2). We iteratively extracted ~1,000,000 particle images from ~9100 micrographs for multi-particle 3D refinement (Supplementary Fig. 3a). Refinement led to two maps for monomeric RNAP-δ-HelD and dimeric (RNAP-δ-HelD)2 complexes at global resolutions of 4.2 and 3.9 Å, respectively, with local resolutions extending beyond these limits (Supplementary Fig. 3b, Supplementary Table 2).

In both monomeric and dimeric complexes, we observed well-defined density for RNAP subunits α1/2 (N-terminal domains [NTDs]), β, β′, δ, ε, and HelD (Supplementary Fig. 4). Density for the ω subunit or nucleic acids was missing. Unless mentioned otherwise, the following descriptions refer to the monomeric complex.

Organization of RNAP in an RNAP-δ-HelD complex. In the RNAP-δ-HelD complex, RNAP adopts a conformation in which the main channel, where downstream DNA and the RNA:DNA hybrid are accommodated in an EC, is wide open, with a distance of ~52 Å between the β2 lobe (P242) and the β′ clamp helices (N283), compared to ~18 Å between the corresponding elements in the E. coli EC31 (Fig. 1a, b and Supplementary Table 3), and a concomitant widening of the RNA exit tunnel by more than 17 Å (β flapR808 to β′ lidD2345). The α1/2NTD dimer remains bound at the closed end of the open β/β′ crab claw.

δ consists of a folded N-terminal domain (NTD; residues 1–90) and an intrinsically disordered acidic C-terminal region (CTR; residues 91–173) with a net ~47 negative charge. As noted previously, the first ~70 residues of δNTD resemble the globular domain of σ1.1 regions of group 1 factors. However, unlike the σ1.1 domain in an E. coli σ34 holoenzyme, δNTD does not reside in the main channel but binds on the surface of RNAP between the β′ shelf and jaw (Fig. 1a and Supplementary Fig. 5a), in agreement with a previous in vivo CLMS analysis. Comparison to the E. coli EC31 showed that δNTD seems to contribute to main channel opening by somewhat contracting the jaw and β′ shelf; furthermore, RNAP opening and slight δNTD-mediated displacement of the shelf lead to the repositioning of β′ secondary channel elements, which would clash with ω at its canonical binding site (Supplementary Fig. 5a), explaining loss of ω in RNAP-δ and RNAP-δ-HelD complexes (Supplementary Fig. 1b, c). Lack of continuous cryoEM density beyond δNTD shows that δCTR is suspended from the rim of the main channel in a flexible manner (see below).

The ε subunit is positioned in a cavity formed by the α1/2 NTDs, the C-terminal β clamp, and β′ residues 492–655 that form part of the secondary channel (Fig. 1a), in contrast to the previous mapping of ε at the β′ jaw based on a low-resolution cryoEM.
analysis and structural similarity of ε to the phage T7 Gp226. Interestingly, in archaeal and eukaryotic nuclear RNAPs this position is occupied by a domain of α1 subunit homologs (Supplementary Fig. 5b). We surmise that ε supports the structural integrity of RNAP, securing interactions between α, β, and β′ subunits when β and β′ are forced apart by HelD.

HelD invades RNAP channels. HelD consists of four domains/regions: an N-terminal region (NTR; residues 4–187), two globular domains (D1a/D1b, residues 188–338/491–603; D2, residues 604–774), and an elongated helical protrusion in D1 (HelDBumper, residues 339–490; Fig. 2a). The NTR exhibits remarkable resemblance to GreA/B transcript cleavage factors, but with an extended coiled-coil (HelDPike, residues 4–96; Figs. 1c and 2b). D1 and D2 resemble NTPase/helicase domains of UvrD36, with a subdomain deleted from D2 and HelDBumper inserted into D1 (Fig. 2c).

HelDBumper lacks close structural similarity to other proteins in the Protein Data Bank (https://www.rcsb.org). HelD is reminiscent of a two-pronged fork poking into RNAP. In perfect analogy to transcript cleavage factors37, one prong, HelDPike, inserts deeply into the secondary channel, through which substrate NTPs enter the RNAP active site during elongation (Fig. 1a, c). D1/D2 reach around the β2 lobe, positioning the other prong, HelDBumper, in the main channel where it pushes against the β′ clamp, forcing β and β′ apart (Fig. 1a). In the course of HelD engaging RNAP, a large combined surface area (~11,500 Å² total; ~8000 Å² with β′; ~1800 Å² with β; ~1700 Å² with δ) is buried.

We observed some cryoEM density patches around HelDBumper that could only be interpreted as parts of δCTR (Fig. 1a). However, the poor quality of the local cryoEM density did not permit reliable modeling of the precise region of δCTR that bound at HelDBumper. We confirmed a direct HelD-δ interaction via δCTR.
**Fig. 2** HelD architecture. **a** Cartoon plot of HelD colored by domains (for color coding see legend). Numbers refer to domain borders. **b** Comparison of HelDNTR to GreB (PDB ID 6RIN) reveals similar topology of the coiled-coils, which insert into the secondary channel, and the globular domains; in GreB, the latter is responsible for high-affinity binding to the RNA β′rim helices. HelDNTR and GreB are rainbow-colored (blue, N-termini; red, C-termini). Numbers refer to domain borders. **c** Comparison of NTPase domains in HelD and in E. coli UvrD (PDB ID 2I56). The D1-D2 regions are rainbow-colored (blue, N-termini; red, C-termini) as indicated in the legends. Neighboring and inserted regions (Ins), gray. Numbers refer to domain borders.

by analytical SEC; while HelD co-migrated with δ and the complex eluted earlier than the individual proteins (Fig. 3a), no such interaction was detected with δNTD (Fig. 3b). These results suggest that δCTR might help position HelDBumper in the main channel, supporting HelD in its push against the β′ clamp (Fig. 1a). HelDBumper and tentatively modeled portions of δCTR reside in a position equivalent to the globular δ′−1 domain in an E. coli σE gene product and a helix following the σα−1 region in a Mycobacterium smegmatis σA holoenzyme34 and a helix following the σα−1 region in a Mycobacterium smegmatis σA holoenzyme38 (Fig. 3c–e). Thus, HelDBumper and δCTR occupy regions next to the β subunit where downstream DNA is accommodated in the EC (Fig. 3c).

Due to the combined actions of δ and HelD, DNAP−δ−HelD exhibits the most open main channel configuration observed in RNAP complexes to date, augmented by about 30 Å and 20 Å relative to the E. coli σE and M. smegmatis σA holoenzymes34,38 respectively (Fig. 3c–e). To confirm contacts and the marked structural rearrangements triggered by HelD binding, we used RNAPΔΔHelD and recombinant δ and HelD to assemble RNAPΔΔHelD−δ, RNAPΔΔHelD−HelD, and RNAPΔΔHelD−δ−HelD, and mapped molecular neighborhoods in these complexes and RNAPΔΔHelD by CLMS with the heterobifunctional, photoactivatable crosslinker sulfo-SDA 4,4′-azipentanoate (sulfo-SDA; Fig. 4a, b and Supplementary Table 4; Supplementary Data 1). Matching the δNTD binding site deduced by cryoEM, a short stretch of δ residues cross-linked to the β′ jaw in both RNAPΔΔHelD−δ (δV63,F83,Y85,K103S) and RNAPΔΔHelD−δ−HelD (δV63,Y85,L87,E90,K103S), Multiple crosslinks of HelD were identified for RNAPΔΔHelD−HelD and RNAPΔΔHelD−δ−HelD complexes inside the RNAP main channel, along the region connecting the main and secondary channels, and in the active site region, in excellent agreement with our cryoEM structures (Supplementary Fig. 6).

RNAPΔΔHelD−RNAPΔΔHelD−δ, and RNAPΔΔHelD−HelD yielded significantly more crosslinks than RNAPΔΔHelD−δ−HelD and, among those, in particular, many more over-length crosslinks when compared to the RNAP−δ−HelD structure (Fig. 4c, d). Furthermore, the fraction of crosslinks corresponding to over-length crosslinks was strongly increased in RNAPΔΔHelD and RNAPΔΔHelD−δ compared to complexes containing HelD (Fig. 4c, d). The reduced total number of crosslinks suggests a reduction in conformations explored by RNAP upon δ or HelD binding, and in particular when both factors are present. The reduced total number and fraction of over-length crosslinks suggest a conformation closer to our RNAP−δ−HelD cryoEM structure in the presence of HelD. A specific set of crosslinks between the β1/2 lobes (residues 146–248) and the β′ shelf (residues 794–1141) represents conformations in which β and β′ approach each other across the main channel unless both δ and HelD are bound to RNAP (Fig. 4e, f). Together, our results demonstrate that HelD interacts with the main and the secondary channels of RNAP and that stable main channel opening depends on the presence of both δ and HelD.

HelDPike dismantles the RNAP active site and competes with RNA. Upon penetrating the secondary channel, HelDPike locally disrupts the β′ bridge helix (BH; between residues 780 and 787) and locks the β′ trigger loop (TL; Fig. 5), i.e., key elements that rearrange for nucleotide addition during elongation35. While HelDPike carries negatively charged side chains (DS6, DS7, E60) at its tip, these residues do not remodel the active site as observed with GreB37. Instead, the tip plows through the active site, thereby dismantling it. The β C-terminal clamp is pushed away from the nucleic acids, β switch region 3 (Sw3), which lines the hybrid in the EC, becomes disordered and the active site loop (ASL) is rearranged so that the catalytic Mg2+ ion is lost (Fig. 5).

RNAP−RNA binary complexes are catalytically active, implying that RNA resides in the active site cavity37. As seen by comparison with an E. coli EC31, the HelDPike tip binds in direct competition to RNA in the hybrid (Fig. 3f) and may additionally repel RNA via the negatively charged residues. Thus, HelDPike rearranges active site regions and spatially competes with all RNAs bound in the vicinity. RNA release would be facilitated by RNA exit tunnel opening via HelDBumper.
subsequently added δ and/or HelD. We first tested displacement of DNA with an artificial bubble, which when bound to RNAP mimics a situation ensuing after many intrinsic termination events. HelD displaced about 25% of DNA from RNAPΔΔΔHelD, while δ led to about 80% displacement in the absence of HelD (Fig. 3g, lanes 4–6). Increasing amounts of δ titrated to DNA-bound RNAPΔΔΔHelD in the presence of stoichiometric amounts of HelD led to a gradual reduction of bound DNA, with essentially all DNA displaced when equimolar amounts of δ relative to RNAPΔΔΔHelD-HelD were added (Fig. 3g, lanes 7–13). Only ~50% of the DNA were displaced by the addition of equimolar amounts of HelD and δNTD (Fig. 3g, lane 14).

Next, we tested the ability of δ/HelD to dissociate ECs assembled on an artificial DNA bubble and complementary RNA, mimicking stalled ECs. A similar picture as for DNA-only
displacement emerged; however, due to the RNA-mediated stabilization of DNA on RNAP, HelD and δ individually or HelD/δNTD liberated less RNA, and higher concentrations of δ in the presence of HelD were required to achieve full nucleic acid displacement (Fig. 3g, lanes 15–28). Notably, δ/HelD-mediated DNA or DNA/RNA displacement did not require the addition of ATP. Together, these results explain why a nucleic acid scaffold failed to associate with the RNAP-δ/HelD complex during preparation for cryoEM; they underscore the importance of δ in nucleic acid displacement, show that HelD is required to achieve complete nucleic acid release and support the cooperation of δCTR and HelD inferred from our structure and CLMS.

ATP-dependent HelD release. As HelD completely incapacitates RNAP (Fig. 5a, b), it has to be released to allow transcription to resume. δA did not displace HelD in SEC (Supplementary Fig. 7a). Comparison of UvrD bound to DNA and ADP-MgF₆ showed that the D1/D2 conformation of RNAP-bound UvrD is incompatible with ATP binding (Fig. 6a). Since ATP binding to HelD induces conformational changes, as revealed by SAXS, we surmised that ATP-bound HelD may have a lower affinity for RNAP than the apo factor. Consistent with this notion, ATPγS, AMP-PNP, and, to a somewhat lesser extent, ATP led to the release of HelD from RNAP-δ/HelD during SEC, while ADP or AMP had minor effects (Fig. 6b and Supplementary Fig. 7b). HelD exhibits intrinsic ATPase activity that is unaltered in the presence of RNAP, thus, AMP-PNP and ATPγS mimic conditions of constantly high ATP supply, whereas ATP is likely hydrolyzed and separated from RNAP/HelD during SEC, reducing its effect. Unlike HelD, δ is not displaced from RNAP by the addition of ATP or analogs (Fig. 6b and Supplementary Fig. 7b).

Dimeric (RNAP-δ/HelD)₂. About two-thirds of our cryoEM particle images conformed to dimeric (RNAP-δ/HelD)₂ complexes (Fig. 6c), which were partially stable during SEC under conditions identical to cryoEM sample preparation (0.15% n-octylglucoside; Supplementary Fig. 1d). We also conducted negative stain EM analyses with RNAP-δ/HelD in the presence or absence of 0.15% n-octylglucoside and detected dimers under both conditions (Supplementary Fig. 1c; a quantitative analysis of the monomer/dimer distribution was precluded by preferred particle orientations on the carbon films). The protomers of the dimeric assembly closely resemble the monomeric RNAP-δ/HelD complex (root-mean-square deviation of 1.2–1.3 Å for 23,360–23,971 pairs of Ca atoms), but elements of the RNAP active site are further remodeled in the dimer (Fig. 5a, b). The HelD-repositioned clamp forms an essential contact region in the dimer, which also involves the initiation/elongation factor-binding β flap tip (FT; Fig. 6c). The dimeric (RNAP-δ/HelD)₂ complex shows a striking resemblance to the hibernating dimeric eukaryotic RNAP, with analogous regions contributing to the dimer interfaces (Fig. 6d). As in (RNAP-δ/HelD)₂, each protomer of the hibernating RNAP I dimer exhibits a wide-open DNA-binding cleft, partially unfolded bridge helix, and a DNA-mimicking loop stably bound inside the cleft, similar to δCTR. Furthermore, the A12.2 C-terminal domain of RNAP I is located inside the secondary channel, representing a dormant state. These observations suggest that, like the RNAP I dimer, dimeric RNAP-δ/HelD may represent a dormant state.

Discussion

Results of this and the accompanying reports show that HelD mounts a two-pronged attack at the RNAP main and secondary channels. Both B. subtilis and the distantly related M. smegmatis HelD pinch RNAP around the BH, widen the main and RNA exit channels to provide escape routes for DNA and RNA, and displace the bound nucleic acids. However, the exact implementation of this conserved mechanism are distinct. B. subtilis HelD uses similarly sized arms to penetrate deeply into the channels, with δ playing a supporting role. δNTD aids the main channel opening, whereas δCTR may support HelD recruitment and guide HelD-Bumper into the main channel to avoid topological trapping of DNA. In contrast, M. smegmatis HelD has evolved a branched main channel arm that functionally compensates for the absence of δ and for a rudimentary secondary channel arm, which merely helps HelD anchoring on RNAP. As HelD and δ did not require ATP addition to displace nucleic acids from RNAP, we presume that the large surface area buried upon RNAP-δ/HelD complex formation, rather than HelD ATPase, provides the driving force for the marked RNAP opening.

To engage RNAP, HelD reaches around the β2 lobe, a mode of attack that is not possible with RNAPs containing a β' lineage-specific insertion, SI3, stacked onto the β2 lobe, such as E. coli (Supplementary Fig. 8a). Consistently, E. coli does not encode HelD, and a distantly related ATPase, RapA, has been proposed to aid RNAP recycling. Unlike HelD, RapA binds near the DNA exit tunnel and does not induce major conformational changes in the EC (Supplementary Fig. 8b). Instead, RapA is thought to rescue ECs by promoting backtracking. Alternative recycling mechanisms likely exist in SI3-containing species. Indeed, E. coli DksA has recently been proposed to remove RNAP from nucleic acids. DksA binds in the secondary channel using a Gre-like coiled-coil, induces conformational changes in RNAP, albeit
less marked than HelD, and is present only in bacteria that have SI330.

Our cryoEM structures also inform about likely mechanisms of action of the δ subunit during initiation and elongation. Previously, δ alone had been shown to displace nucleic acids from RNAP7, a result we recapitulate here (Fig. 3g). As δCTR peptides showed similar activity when added in excess and as δNTD was found to bind RNAP, δ-mediated nucleic acid displacement was suggested to involve δNTD-dependent tethering of the polyanionic δCTR to core RNAP. Our cryoEM structures confirm and further refine this hypothesis. δNTD anchors δCTR at the rim of the main channel; due to its length and intrinsic disorder, δCTR can reach
into and explore most of the volume of the main channel, spatially and electrostatically competing with bound nucleic acids. This mode of action would explain how δ enhances core RNAP recycling in multi-round assays, and it may constitute the main recycling mechanism in bacteria that contain δ but lack HeD. It also provides an explanation for the finding that in vitro, RNAP-δ in the presence of σ factors still binds promoters and forms closed complexes but fails to establish contacts with the downstream DNA, which are required for the transition to an open complex. Finally, the model suggests that abolishing a positively charged region at the δCTR N-terminus, to promote more extended conformations of the CTR, effectively abrogates a restraint on δCTR to invade the main channel, thus reconciling increased effects of such CTR variants at promoters that form unstable complexes.

δ exhibits negative cooperativity with σA and favors its exchange for alternative σ factors that lack σ11. In the E. coli σ34 holoenzyme, σ11 can reside in the main channel, preventing access of either double- or single-stranded DNA to the RNAP active site (Fig. 3d). To allow for DNA loading, the clamp has to open further or σ11 has to move. These observations suggest competition between δ and σ11 in full, with σA in our structures (Fig. 3c–e). However, while δNTD resembles the globular domain of σA1-32, our results indicate that the structurally unrelated CTR (together with HeDBuffer, if present) constitutes the σ11-competitive element that can occupy equivalent regions in the main channel (Fig. 3c).

The HeD/δ-dependent recycling mechanism uncovered here represents a marvelously simple, direct, and effective way of recovering RNAP from virtually any state trapped post-termination. However, RNAP is truly recycled only when HeD also detaches. We show that HeD is released by ATP (Fig. 6b and Supplementary Fig. 7b), suggesting that high levels of ATP could help prevent HeD from trapping RNAP in an inactivated complex during exponential growth. Noteworthy, both B. subtilis and M. smegmatis HeDδs cannot bind ATP when fully engaged with RNAP, suggesting that intrinsically timed isomerization into a less engaged conformation must precede ATP binding and release from RNAP. Irrespective, we suggest that ATP-mediated HeD release underlies the ATP-dependent stimulatory effect of HeD on transcription. In contrast, ATP does not induce the concomitant release of δ (Fig. 6b and Supplementary Fig. 7b), confirming that δ has an intrinsically high affinity for RNAP and does not require HeD to remain stably associated. As an association of alternative σ factors (relative to σA) is favored in RNAP-δ compared to RNAP lacking δ, additional mechanisms may be at play to remove δ (or expunge δCTR from the main channel) in situations where efficient rebinding of σA is specifically required.

When cells sporulate during the stationary phase, conversely, the levels of ATP are low, transcription is limited, HeD levels match those of RNAP, and HeD is thus expected to remain bound to RNAP. Given that HeD locks RNAP in an inactive state, could it be used to store RNAP until the conditions improve? Intriguingly, we observed (RNAP-δ-HeD)2 dimers resembling hibernating ε-coreycteric RNAP I (Fig. 6c, d), which were partially stable in SEC at initial RNAP concentrations about 10-fold lower compared to their nominal cellular concentrations in the log phase, estimated from transcript levels and ribosome profiling. Dimerization of RNAP has also been reported in bacteria that lack HeD, including E. coli. While dimerization may be an inherent property of RNAPs, our results clearly show that HeD, while not directly involved in forming the dimer interface, facilitates the observed mode of dimerization by pushing the β′ clamp outwards to enable homologous contacts between the β′ clamps, the C-terminal β clamp, and regions of the β flap (Fig. 6c). Notably, a comparison of our dimeric structure to an M. smegmatis RNAP-σA holoenzyme structure shows that all binding sites for σ, except for σ11 in the main channel, would be accessible in the RNAP-δ-HeD dimer. Thus, rebinding of σ could contribute to the efficient recovery of RNAP from the dimeric state. Taken together, HeD/δ could in principle promote RNAP hibernation that may be essential for fast RNAP recovery, in line with observations that overexpression of HeD enhances sporulation and deletions of HeD, δ or both prolong the lag phase. Further tests of this idea are required and could involve in vivo CLMS at different growth phases and during sporulation, in WT compared to ΔheLD or overexpressing cells, or in vivo super-resolution imaging with fluorescence labeling of HeD or RNAP.

This and the accompanying studies present a hitherto unrecognized transcription recycling system that safeguards genome integrity and may contribute to persistence during periods of dormancy. In our model, parts of which require further validation, reservoirs of active RNAP are controlled by HeD, which may rescue trapped RNAP during fast growth, promote RNAP hibernation during slow growth, and enable efficient RNAP recovery upon shift to a nutrient-rich environment. We note that although most laboratory experiments are carried out with rapidly growing bacteria for convenience, dormant states are prevalent in natural environments and pose grave health risks. For example, B. anthracis spores are the infectious particles for anthrax, whereas slow-growing Pseudomonas aeruginosa biofilms and M. tuberculosis are resistant to cidal antibiotics. Unraveling the regulation of dormancy is thus critical for the understanding of bacterial physiology and identifying new strategies for the eradication of multidrug-resistant pathogens.

Methods

Plasmids, DNAs, and RNAs. A DNA fragment encoding B. subtilis HeD was PCR-amplified from strain MH5636 (Supplementary Table 1). The PCR product

Fig. 4 Structure probing by CLMS. a Map of hetero-protein crosslinks observed in RNAPΔΔHelD-δ-HeD complex. Crosslinks identified in RNAPΔΔHelD, RNAPΔΔHelD-δ, RNAPΔΔHelD-HeD, and RNAPΔΔHelD-δ-HeD. Binding of both δ and HeD leads to strongly reduced crosslinking between β and β′. c Distribution of Ca-Ca distances between crosslinked residue pairs in reference to the RNAP-δ-HeD structure. Crosslinks with Ca-Ca distances within 25 Å, the theoretical crosslinking limit of sulfu-SDA, green; crosslinks with Ca-Ca distances >25 Å, magenta; distance distribution of random residue pairs in the RNAP-δ-HeD structure, gray. d Numbers of crosslinks (bars) between β and β′ identified from the four cross-linked complexes, and fractions of over-length crosslinks (percentages at the bottom). Crosslinks are color-coded as in b. In the RNAPΔΔHelD-δ-HeD complex, a significantly reduced number of β-β′ over-length crosslinks (in reference to the RNAP-δ-HeD structure) compared to the RNAPΔΔHelD, RNAPΔΔHelD-δ, and RNAPΔΔHelD-δ-HeD complexes suggests that δ and HeD cooperate to stabilize an open conformation of RNAP. e Comparison of β-β′ crosslinks observed with RNAPΔΔHelD, RNAPΔΔHelD-δ, RNAPΔΔHelD-HeD, and RNAPΔΔHelD-δ-HeD. The green boxed region, crosslinks between the β/2 lobes (residues 146–248) and the β′ shell and jaw (residues 794–1141) observed in the first three complexes but almost absent in RNAPΔΔHelD-δ-HeD. f Structure of the RNAP-δ-HeD complex highlighting the β′/2 lobes (lemon green) and β′ shell and jaw (forest green), which largely lack crosslinks in the presence of δ and HeD (green box in e).
**Protein purification and preparation.** *B. subtilis* strains MH5636, LK782 (ΔhelD) or LK1032 (ΔhelD/FliJ; Supplementary Table 1) were used to produce stationary phase RNAP, RNAPΔδNTD or RNAPΔδΔNTD, respectively. In these strains the chromosomally-encoded pβ subunit carries a C-terminal His10-tag. Strains were grown in TB medium at 37 °C to an OD600 of 1.0 and were then shifted to 18 °C and grown to an OD600 of about 11. All purification steps were performed at 4 °C. Cells were harvested by centrifugation, resuspended in buffer A (50 mM Na2HPO4, 300 mM NaCl, 4 mM HEPES, 80 °C), and lysed by sonication. The lysate was cleared by centrifugation. RNAP variants were captured on Ni2+-NTA affinity resin (Macherey-Nagel), washed with buffer A supplemented with 25 mM imidazole, and eluted with buffer A supplemented with 250 mM imidazole. The eluate was dialyzed overnight against 50 mM Na2HPO4, 300 mM NaCl, 3 mM DTT, 5% [v/v] glycerol, pH 7.9, loaded on a 5 ml HiTrap Heparin HP column (GE Healthcare), washed with buffer B (50 mM TRIS-HCl, 100 mM NaCl, 3 mM DTT, 0.1 mM EDTA, 5% [v/v] glycerol, pH 7.9) and eluted with a linear gradient to buffer B with 700 mM NaCl. Fractions containing RNAPs were pooled and further purified by SEC on a HiLoad Superdex 200 Increase 16/600 column (GE Healthcare) in 20 mM TRIS-HCl, 150 mM NaCl, 0.5 mM DTT, 5% [v/v] glycerol, pH 8.0. The final samples were concentrated to approximately 16 mg/ml. RNAP produced from strain MH5636 was directly used for EM sample preparation. Other RNAP preparations were aliquoted, flash-frozen in liquid N2, and stored at −80 °C.

Recombinant *B. subtilis* GST-HelD was produced in *E. coli* Rosetta(DE3) cells, His6-δ, His6-δNTD, and His6-δNTC were produced in *E. coli* BL21(DE3)-RIL cells. Cells were grown in auto-inducing media at 37 °C to an OD600 of 1.0 and further incubated at 20 °C overnight. All purification steps were performed at 4 °C. GST-HelD cells were harvested by centrifugation, resuspended in buffer C (50 mM TRIS-HCl, 500 mM NaCl, 1 mM 2-mercaptoethanol, 10% [v/v] glycerol, pH 7.9), and lysed by sonication. The lysate was cleared by centrifugation, GST-HelD was captured on glutathione resin (Macherey-Nagel), washed with buffer C, and eluted with 50 mM TRIS-HCl, 300 mM NaCl, 1 mM DTT, 10% [v/v] glycerol, 20 mM reduced glutathione, pH 7.9. Eluted fractions were dialyzed against buffer D (20 mM TRIS-HCl, 200 mM NaCl, 1 mM DTT, 5% [v/v] glycerol, pH 7.9) in the presence of GST-tagged PreScission protease. HelD was separated from uncleaved protein, GST, and GST-PreScission by a second passage through glutathione resin. The flowthrough was further purified by SEC on a HiLoad Superdex 200 Increase 16/600 column equilibrated in buffer D. Fractions containing HelD were concentrated to approximately 15 mg/ml, aliquoted, flash-frozen in liquid N2, and stored at −80 °C.

His6-δ or His6-δNTD cells were harvested by centrifugation, resuspended in 50 mM TRIS-HCl, 500 mM NaCl, 0.5 mM 2-mercaptoethanol 5% [v/v] glycerol, pH 6.0, and lysed by sonication. The lysate was cleared by centrifugation, His6-δ/His6-δNTD was captured on Ni2+-NTA resin, washed with 30 mM TRIS-HCl, 0.5 mM NaCl, 0.5 mM 2-mercaptoethanol, 10 mM imidazole, 5% [v/v] glycerol, pH 6.0, and eluted with 20 mM TRIS-HCl, 150 mM NaCl, 0.5 mM 2-mercaptoethanol, 5% [v/v] glycerol, pH 6.0. For the assembly of complexes for cryoEM analysis, eluted His6-δ was supplemented with His-tagged TEV protease (1:40 [w/w]), dialyzed against buffer E (20 mM TRIS-HCl, 150 mM NaCl, 1 mM DTT, 5% [v/v] glycerol, pH 6.0) overnight and passed through fresh Ni2+-NTA resin to remove the uncleaved His6-δ, the cleaved His-tag, and His-tagged TEV protease. Proteins were further purified by SEC on a Superdex 75 Increase 10/300 column (GE Healthcare) in buffer E. Fractions containing His6-δ, His6-δNTD or His6-δNTC were concentrated to ~4 mg/ml (His6-δ, His6-δNTD and 23 mg/ml (δ)), aliquoted, flash-frozen in liquid N2, and stored at −80 °C.

His6-δ or His6-δNTD cells were harvested by centrifugation, resuspended in buffer F (20 mM TRIS-HCl, 500 mM NaCl, 1 mM 2-mercaptoethanol, 5% [v/v] glycerol, pH 7.5) supplemented with 20 mM imidazole, and lysed by sonication. The lysate was cleared by centrifugation, His6-δ was captured on Ni2+-NTA resin, washed with buffer F supplemented with 50 mM imidazole, and eluted with buffer F supplemented with 400 mM imidazole. Eluted His6-δ was supplemented with His-tagged TEV protease (1:40 [w/w]), dialyzed against buffer F supplemented with 1 mM EDTA overnight, and passed through fresh Ni2+-NTA resin to remove uncleaved His6-δ, cleaved His-tag and His-tagged TEV protease. The target protein was further purified by SEC on a Superdex 75 Increase 16/600 column (GE Healthcare) in 25 mM TRIS-HCl, 300 mM NaCl, 0.1 mM DTT, 5% [v/v] glycerol, pH 7.5. Fractions containing His6-δ were concentrated to approximately 39 mg/ml, aliquoted, flash-frozen in liquid N2, and stored at −80 °C.

Crosslinking/mass spectrometry. Sulfo-SDA predominantly establishes lysine-X crosslinks through a primary amine-reactive moiety on one side and a UV-activatable moiety on the other (theoretical crosslinking limit 25 Å). Sulfo-SDA was prepared at 3 mg/ml in 20 mM HEPES-NaOH, 5 mM Mg(OAc)2, 5 mM DTT, 5% [v/v] glycerol, pH 8.0 immediately prior to addition of RNApδ-ΔHelD, RNAPδNTDΔHelD, HelD, or RNAPδΔHelD-ΔHelD (protein: sulfo-SDA Δ3 [w/w]). Samples were incubated on ice for two hours and then irradiated in a thin film using 365 nm UV (UVCL-1000 UVCrosslinker, UVB Inc.) for 20 min on ice (5 cm distance from UV-A lamp). The crosslinked samples were separated by 4–12% Bis-Tris NuPAGE gels. The crosslinking monomeric complexes were excised and digested in-gel[86]. The resulting peptides were desalted using C18 StageTips[81].
**Fig. 6 HelD release and RNAP-HelD complex dimerization.**

a Close-up view of the ATP-binding site of HelD, with ADP-Mg\(_2\)F\(_3\) from a UvrD complex (PDB ID 2IS6) transferred by superpositioning of the UvrD NTPase domains on HelD, illustrating clashes with the nucleotide. ADP-Mg\(_2\)F\(_3\) shown as sticks and colored by atom type; carbon, beige; nitrogen, blue; oxygen, red; phosphorus, orange; magnesium ions, green; fluoride ions, light blue.

b SDS-PAGE analysis of SEC runs after treating RNAP-δ-HelD with buffer or the nucleotides indicated on the left. Experiments were repeated independently at least twice with similar results. For full gels, see Supplementary Fig. 7b.

c Structure of dimeric RNAP-δ-HelD. Inset, close-up view on the dimer interface. The two protomers interact via the elements highlighted in colors; β′ clamp, violet; C-terminal β clamp, green; β flap tip (FT), olive; residue 811–821 of the β flap (Flag\(^{811-821}\)), yellow.

d Structure of a hibernating RNAP I dimer (PDB ID 4C2M). A135 subunit, black; A190 subunit, white; small subunits, beige. Inset, close-up view on the dimer interface. A190 clamp, violet; C-terminal A135 clamp, green.
1° mediated recovery from the dormant state represent tentative aspects of the model. The ions with a charge state from 3 to 5 were isolated and fragmented using higher-energy collisional dissociation (HCD) with 30% collision energy. The fragmentation spectra were then recorded in the Orbitrap with a resolution of 120,000. The ions with a charge state ranging from 6 to 7 were isomerized and fragmented using xiFDR64. A false discovery rate of 5% on the residue-level was applied with the xiFDR method to identify crosslinked peptides. The cross-linking of RNAPδΔδ-HelD, HelD, and RNAPδΔδ-HelD–Δ-HelD samples, protein sequences of δ, HelD or both were included in the database. The following parameters were applied for the search: MS accuracy = 4 ppm; MS2 accuracy = 8 ppm; enzyme = trypsin (with full trypsin specificity); allowed number of missed cleavages = 2; missing monoisotopic peak = 2; crosslinker = sulfo-SDA (the reaction specificity for sulfo-SDA was assumed to be for lysine, serine, threonine, tyrosine, and protein N-termini on the NHS ester end, and any amino acid residue for the diazirine end); fixed modifications = carboxymethylation on cysteine; variable modifications = oxidation on methionine and sulfo-SDA loop link. Identified crosslinked peptide candidates were filtered using xiFDR64. A false discovery rate of 5% on the residue-pair level was applied with the “boost between” option selected. Crosslinked residue pairs identified from the four complexes are summarized in Supplementary Table 4 and Supplementary Data 1.

CryoEM sample preparation, data collection, and processing. Equimolar amounts of tDNA, ntDNA, and RNA were mixed in buffer G (20 mM TRIS-HOAc, 5 mM Mg[OAc]2, 100 mM KOAc, 2 mM DTT, 5% [v/v] glycerol, pH 8.0) for 10 min on ice, then for 10 min at 32 °C. Equimolar amounts (to RNAPδ) of δ and HelD were added stepwise, followed by incubation for 10 min at 32 °C after each addition. The mixture was subjected to LC-MS/MS data generated from the four complexes were processed separately. MS2 peak lists were generated from the raw MS data files using the MSConvert module in ProteoWizard (version 3.0.11729). The default parameters were applied, except that Top MS/MS Peaks per 100 Da was set to 20 and the denoising function was enabled. Precursor and fragment m/z values were recalibrated. Identification of cross-linked peptides was carried out using xiSEARCH software (https://www.rappsilberlab.org/software/xiSEARCH; version 1.7.4)65. For RNAPδΔΔHeID, peak lists were searched against the sequence and the reversed sequence of RNAP subunits (α, β, β′, ε, and σ) and two co-purified proteins, α6 and β6. For RNAPαδΔΔHeID, RNAPβΔΔΔHeID, HelD, and RNAPδΔΔHeID–Δ-HelD samples, protein sequences of δ, HelD or both were included in the database. The following parameters were applied for the search: MS accuracy = 4 ppm; MS2 accuracy = 8 ppm; enzyme = trypsin (with full trypsin specificity); allowed number of missed cleavages = 2; missing monoisotopic peak = 2; crosslinker = sulfo-SDA (the reaction specificity for sulfo-SDA was assumed to be for lysine, serine, threonine, tyrosine, and protein N-termini on the NHS ester end, and any amino acid residue for the diazirine end); fixed modifications = carboxymethylation on cysteine; variable modifications = oxidation on methionine and sulfo-SDA loop link. Identifed crosslinked peptide candidates were filtered using xiFDR64. A false discovery rate of 5% on the residue-pair level was applied with the “boost between” option selected. Crosslinked residue pairs identified from the four complexes are summarized in Supplementary Table 4 and Supplementary Data 1.

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SEC on a Superdex 200 Increase 3.2/300 column (GE Healthcare) in buffer H. Fractions containing RNAP, δ, and HelD were pooled and concentrated to approximately 5 mg/ml.

Immediately before preparation of the grids, the sample was supplemented with 0.15% (w/v) n-octylglucoside (critical micellar concentration 0.6% [w/v]). 3.8 µl of the final mixture were spotted on plasma-treated Quantifoil R1/2 holey carbon grids at 100°C/100% humidity and plunged into liquid ethane using an FEI Vitrobot Mark IV. Image acquisition was conducted on an FEI Titan Krios G3 (300 kV) transmission electron microscope (TEM) with a Falcon 3ECC camera at a nominal magnification of 92,000× in counting mode using EPU software (ThermoFisher Scientific) with a calibrated pixel size of 0.832 Å. A total electron dose of 40 e⁻/Å² was accumulated over an exposure time of 56 s. Movie alignment was done with MotionCor265 using 5 × 5 patches followed by ctf estimation with Gctf66.

All following image analysis steps were done with cryoSPARC76. Class averages of manually selected particles were used to generate an initial template for reference-based particle picking from 9127 micrographs. Particle images were extracted with a box size of 440 and binned to 110 for initial analysis. Ab initio reconstruction using a small subset of particles was conducted to generate an initial 3D reference for 3D heterogeneous refinement. The dataset was iteratively classified into two well-resolved populations representing monomeric and dimeric RNAP-δ-HelD. Selected particles were re-extracted with a box of 220 and again classified in 3D to further clean the dataset. Finally, selected particle images were re-extracted with a box of 280 (1.3 Å/px) and subjected to local refinement using a generously enlarged soft-mask for monomeric or dimeric RNAP-δ-HelD. Local refinement of the dimer particles using the monomeric mask was conducted as a control to trace differences of RNAP-δ-HelD in the authentic monomer and dimer structures. After per-particle CTF correction, non-uniform refinement was applied to generate the final reconstructions.

Model building and refinement. The final cryoEM map for the dimeric RNAP-δ-HelD complex was prepared for cryoEM analysis, diluted to 25 µg/ml in buffer H and supplemented with 0.15% n-octyl-glucoside, pre-incubated with 0.05% Tween 20 and further diluted 1:10 with 0.1 M NaCl, 2 mM DTT, pH 7.5, and incubated for 10 min at room temperature. 50 µl of the samples were loaded on a Superdex 200 Increase PC 3.2 column (GE Healthcare) and chromatographed at 4°C with a flow rate of 40 µl/min. Fractions were analyzed by 12.5% SDS-PAGE.

Nucleic acid displacement assays. Equimolar amounts of 5′-[32P]-labeled tDNA and unlabeled tDNA capable of forming an artificial bubble, or additionally an RNA 9-mer with complementarity to the tDNA in the bubble (Supplementary Table 1), were mixed in buffer G and annealed by heating to 95 °C for 5 min and subsequent cooling to 25°C at 1°C/min. The labeled DNA duplex or DNA/RNA scaffold and RNAPδHelD (10 nM and 1 µM final concentrations, respectively) were incubated in buffer G for 10 min at 4°C, followed by an additional 10 min incubation at 32°C. Subsequently, (i) buffer, (ii) HelD (1 µM final concentration); (iii) δ (1 µM final concentration), (iv) combinations of HelD (1 µM final concentration) and δ (titrated final concentration; Fig. 3g) or (v) He1D and δHelD (1 µM final concentration each), and were added, and the samples were further incubated for 10 min at 32°C. Samples were loaded on a 4% native PAGE gel and electrophoresed in 0.5X TBE buffer. Radiolabeled bands were visualized using a Storm phosphorimager and quantified using ImageQuant software (GE Healthcare).

HelD release assays. Equimolar amounts of HelD and stationary phase RNAP were mixed in buffer I (20 mM TRIS-HCl, 300 mM NaCl, 2 mM DTT, 5% (v/v) glycerol, pH 8.0), incubated for 10 min on ice and then for 10 min at 32°C. The sample was chromatographed on a HiLoad Superdex 200 Increase 10/300 column (GE Healthcare) in buffer I. Fractions were analyzed by 12.5% SDS-PAGE, fractions containing RNAP-HelD complex were collected and concentrated to ~3 mg/ml. 80 µl of this complex were mixed with buffer I, 5 mM Mg²⁺-ATP/AMPPNP/ATP/ADP/AMP, 6.7 µM δ or δ plus Mg²⁺-ATP in buffer I. 90 µl of the samples were loaded on a 0.2 µm filter, added to glow-discharged Formvar/carbon grids (S162, Plano GmbH), left to settle for 40 s and manually blotted with Whatman paper No. 1, followed by addition of 0.1% (w/v) uranyl acetate staining solution. After 40 s incubation, the grids were manually blotted and dried at ambient temperature overnight. Samples were imaged on an FEI Talos L120C TEM, operated at 120 kV, equipped with an FEI CETA 16 M CCD camera at a nominal magnification of 57,000×. The calibrated pixel size was 2.53 Å/px. Images were acquired manually in low dose mode using TEM Imaging & Analysis (TIA) software, supplied by the manufacturer, accumulating a total electron dose of 5 e⁻/Å². Image analysis was done with cryoSPARC. After CTF estimation, manually selected particle images were used as a reference for template-based particle picking. Particle images were extracted with a box size of 160 px and resampled to 80 px. A mask of 220 Å diameter was applied to the RCSB Protein Data Bank (https://www.rcsb.org/) with accession codes 6ZCA74 (monomeric RNAP-δ-HelD) and 6ZBF75 (dimeric RNAP-δ-HelD). CLSM data have been deposited in JPOST (https://jpostdb.org/) with accession code JPST000858 (PXID PXD019437)76. Other data are available from the corresponding author upon reasonable request.

Negative stain EM analysis. RNAP-δ-HelD complex was prepared as for cryoEM analysis, diluted to 25 µg/ml in buffer H and supplemented with 0.15% n-octyl-glucoside, pre-incubated with 0.05% Tween 20 and further diluted 1:10 with 0.1 M NaCl, 2 mM DTT, pH 7.5, and incubated for 10 min at room temperature. 50 µl of the samples were loaded on a Superdex 200 Increase PC 3.2 column (GE Healthcare) and chromatographed at 4°C with a flow rate of 40 µl/min. Fractions were analyzed by 12.5% SDS-PAGE.

Model building and refinement. The final cryoEM map for the dimeric RNAP-δ-HelD complex was prepared for initial model building. Coordinates of M. smegmatis RNAP α, β, and β′ subunits (PDB ID 5VB6)86 were docked into the cryoEM map using Coot92. Modeling of δ was based on the NMR structure of B. subtilis δ (PDB ID 2MAK)93. Modeling of ε was supported by the structure of YkzG from Bacillus subtilis δ using 0.025 (w/v) n-octylglucoside, supplemented with 0.025 (w/v) NaN₃, at 18°C with a flow rate of 0.6 ml/min. Data were analyzed with the ASTRA 6.2 software (Wyatt Technology) using monomeric bovine serum albumin (Sigma-Aldrich) as a reference.

Interaction assays. HelD interactions with δ or δSTD were analyzed by analytical SEC. 21 µM HelD and 42 µM δ or δSTD were mixed in 20 mM HEPES-NaOH, 50 mM NaCl, 1 mM DTT, pH 7.5, and incubated for 10 min at room temperature. 50 µl of the samples were loaded on a Superdex 200 Increase PC 3.2 column (GE Healthcare) and chromatographed at 4°C with a flow rate of 40 µl/min. Fractions were analyzed by 12.5% SDS-PAGE.

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Data availability

CryoEM maps have been deposited in the Electron Microscopy Data Bank (https://www.ebi.ac.uk/pdb/emdb/) under accession codes EMD-11104 (monomeric RNAP-δ-HelD) and EMD-11105 (dimeric RNAP-δ-HelD). Structure coordinates have been deposited in the Protein Data Bank (https://www.rcsb.org) with accession codes 6ZCA74 (monomeric RNAP-δ-HelD) and 6ZBF75 (dimeric RNAP-δ-HelD). CLSM data have been deposited in JPOST (https://jpostdb.org/) with accession code JPST000858 (PXID PXD019437). Other data are available from the corresponding author upon reasonable request.

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Author contributions

H.-H.P. cloned genes, produced proteins/complexes and performed experiments with help from Y.-H.H., Y.G., and N.S., prepared cryoEM samples with T.H., built atomic models with B.L. and M.C.W., refined structures with B.L. and performed crosslinking with Z.A.C. T.H., acquired, processed, and refined cryoEM data. Z.A.C. performed CLMS analyses. All authors contributed to the analysis of the data and the interpretation of the results. M.C.W., I.A., and G.B. wrote the manuscript with contributions from the other authors. J.R. and M.C.W. supervised work in their respective groups. M.C.W. conceived and coordinated the project.

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Competing interests

The authors declare no competing interests.

Additional information

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