Characterization of a toxin-antitoxin system in *Mycobacterium tuberculosis* suggests neutralization by phosphorylation as the antitoxicity mechanism

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*Mycobacterium tuberculosis* (Mt) encodes an exceptionally large number of toxin-antitoxin (TA) systems, supporting the hypothesis that TA systems are involved in pathogenesis. We characterized the putative Mt *Rv1044-Rv1045* TA locus structurally and functionally, demonstrating that it constitutes a bona fide TA system but adopts a previously unobserved antitoxicity mechanism involving phosphorylation of the toxin. While *Rv1045* encodes the guanylyltransferase TglT functioning as a toxin, *Rv1044* encodes the novel atypical serine protein kinase TakA, which specifically phosphorylates the cognate toxin at residue S78, thereby neutralizing its toxicity. In contrast to previous predictions, we found that *Rv1044-Rv1045* does not belong to the type IV TA family because TglT and TakA interact with each other as substrate and kinase, suggesting an unusual type of TA system. Protein homology analysis suggests that other COG5340-DUF1814 protein pairs, two highly associated but uncharacterized protein families widespread in prokaryotes, might share this unusual antitoxicity mechanism.
**Mycobacterium tuberculosis** (MtB) is one of the most lethal bacterial pathogens threatening humanity in the 21st century. One striking feature of MtB is that it has an exceptionally large number of toxin-antitoxin (TA) loci. At least 88 TAs were identified in MtB. In stark contrast, a harmless relative of MtB, *M. smegmatis*, encodes only 5 TA systems. This gave rise to the assumption that the TA system may play an important role in pathogenicity. Deep and colleagues reported that the VapBC11 TA system is essential for establishing MtB infection in vivo, providing an example of the TA system contributing to MtB virulence. Whether TA systems contribute to the formation of bacterial persisters cells is currently under debate. While some studies contradict the involvement of TA systems in persisters cells formation in *E. coli*,[8-10], *S. aureus* and *S. enterica*,[11,12] there is evidence supporting the link between TA systems and persisters cell formation in other bacteria.[13-15]. 10 TA modules were found to be overexpressed in the MtB persisters cells,[16] hinting a possible role of TA systems in MtB persisters formation. MtB TAs are considered promising anti-TB drug targets.[17,18] Molecules that disrupt the type II or III toxin-antitoxin complex may act as novel antimicrobial agents.[19] For example, based on the structure of the VapBC26 TA complex, toxin-mimicking peptides were designed. The peptides can activate the toxic function of the toxin by disrupting TA complex formation,[20] thus providing a strategy for developing novel antibiotics.

Six TA families have been identified.[21] Type I system contains a toxin protein and a small antisense RNA that specifically targets the mRNA of the toxin, thus inhibiting its expression. Type II system, in which both toxin and antitoxin are proteins, is the most common and abundant TA systems. The unstable antitoxin deactivates the toxin via forming a stable protein complex with the toxin. In type III system, the toxin protein is directly bound by an RNA molecule acting as the antitoxin. Type IV system also encodes two protein components; however, in contrast with the type II TA, they do not interact with each other. Instead, both toxin and antitoxin act on the same target, however they prompt opposing outcomes. Type V system includes an endoribonuclease as the antitoxin, which degrades the mRNA of the toxin, effectively down-regulating its expression.[22] Type VI TA system encompasses an atypical SocAB TA system identified in *C. crescentus*. The labile toxin ScoB is neutralized by ClpXP proteinase degradation that is assisted by the antitoxin ScoA acting as the proteolytic adaptor.[23]

Among numerous TA systems encoded by the MtB H37Rv strain, most are type II members, including the VapBC, MazEF, YelM/YoeB, ReiBE, HigBA and ParDE families, as well as the tripartite type I TAC (Toxin-Antitoxin-Chaperone) system. Among the remaining 11 uncharacterized TAs, *Rv0836c-Rv0837c, Rv1045-Rv1045 and Rv2827c-Rv2826c* were predicted to be type IV TAs.[24] One well-characterized type IV TA is the YeeU/CbtA module from *E. coli*.[25] The antitoxin YeeU acts against the toxic activity of toxin CbtA like a canonical TA system, however the toxin and the antitoxin do not interact with each other. Instead, CbtA and YeeU both act on the assembly of FtsZ and MreB filaments. While YeeU promotes filament formation, CbtA inhibits it. Thus, the lack of toxin-antitoxin interaction becomes a hallmark of the type IV family. Recently, a type IV TA family member AbiE from bacterial abortive infection systems was identified.[26] However, the interaction between AbiEii and AbiEi is undetectable, which classifies AbiE as a type IV TA. AbiEii is a putative nucleotidyltransferase (NTase) belonging to the DNA polymerase β superfamily, which preferentially binds GTP; thus, it is a guanylyltransferase. Four conserved motifs (I–IV) were identified in AbiEi, the mutations of which impaired or abolished GTP binding activity and toxicity. AbiEii is comprised of an N-terminal winged-helix-turn-helix (WHH) domain and a C-terminal uncharacterized domain (CTD). The N-terminal whHH recognizes a DNA cassette of the abiE promoter repressing its own transcriptional level, whereas the CTD is an uncharacterized domain responsible for neutralization of the toxicity of AbiEii.

AbiE TA and the putative MtB type IV TAs share extensive homology. They all belong to a widely spread and highly associated gene pairs COG5340-COG2253.[27] In which, a gene encoding an NTase acting as the toxin is always followed by a gene encoding a transcriptional regulator acting as the antitoxin. It is worth noting that COG2253 belongs to the large protein superfamily DUF1814, which is ubiquitous not only in bacteria but also in archaea and fungi. However, these abundant protein pairs are largely uncharacterized and the interplay between the toxin and antitoxin remains elusive.

Here, we characterized structurally and functionally *Rv1044-Rv1045* system, a putative type IV TA system from MtB. We found that while *Rv1045* encodes the guanylyltransferase TglT (unusual type guanylyltransferase-like toxin), which arrests bacterial growth, *Rv1044* encodes the atypical protein kinase TakA (unusual type of atypical kinase antitoxin), which neutralizes the activity of TglT via phosphorylation. TglT and TakA interact with each other directly; thus, they do not belong to type IV TA family. Instead, it is an unusual type of TA system, because the anti-toxicity mechanism involving the phosphorylation of the toxin identified in this study has not been observed previously.

**Results**

- **Rv1044 and Rv1045 constitute a TA system.** *Rv1044* (anti-toxin) and *Rv1045* (toxin) are placed under the same operon in the MtB H37Rv genome (Fig. 1a). The 3’ end of *Rv1044* overlaps with the 5’ end of *Rv1045* by 3 nucleotides, an arrangement resembling the bicistronic abiEi/abiEii operon.[28] *Rv1044* was predicted to be essential in MtB based on the Tn library screening,[29] whereas comprehensive essentiality analysis of the MtB genome suggested that *Rv1045* might be toxic in cells.[28]

- To verify *Rv1045* toxicity and *Rv1044* antitoxicity, we inserted the toxin into the L-arabinose inducible expression vector pBAD33 (TglT-His) and the antitoxin into the IPTG inducible expression vector pET28a (His-TakA). In toxicity and antitoxicity assay, the bacterial growth was examined (Fig. 1b). The expression of the His-TakA alone did not lead to growth arrest. On the contrary, colonies could not form when TglT-His was expressed. When the expression of both TglT-His and His-TakA was induced, the toxicity of TglT was neutralized (Fig. 1b), implying that TakA counteracted the toxicity of TglT. This result indicated that *Rv1044-Rv1045* constitutes a TA system. To obtain more details, we recorded the time course of bacterial growth (Fig. 1c). When the toxin was induced first, bacteria growth could be rescued by the induction of the antitoxin later. It is worth noting that the toxicity of the toxin could still be neutralized when the Taka-expressing vector was available but was not induced (Fig. 1c green curve). This was likely due to the “leaking phenomenon” of the T7 system,[30] in which the antitoxin was expressing at a low level despite the absence of IPTG. In the following sections, we will demonstrate that the antitoxin TakA is a kinase that neutralizes the toxin via direct phosphorylation of the toxin, which offers a plausible explanation for this phenomenon: as an enzyme, the antitoxin could be very effective even when expressed at a low level.

To investigate whether *Rv1044-Rv1045* belongs to the type IV TA, we studied the direct interaction between TglT and TakA. We engineered a FLAG-tag at the C-terminus of TglT (TglT-FLAG) and a Myc-tag to the C-terminus of TakA (TakA-Myc). TglT-FLAG and TakA-Myc were co-expressed in BL21 cells and
the expression of both proteins was detectable by western blotting (Fig. 1d). In co-immunoprecipitation (Co-IP) experiment, we used anti-FLAG magnetic beads to pull-down TglT-FLAG as bait, we observed the presence of TakA-Myc (prey). However, we found that TakA-Myc bound to anti-FLAG beads nonspecifically because TakA-Myc was also pulled down in the absence of TglT-FLAG. Therefore, we chose anti-Myc magnetic beads for Co-IP experiment. We observed the presence of TakA-Myc (prey) when we used anti-FLAG magnetic beads to pull down TglT-FLAG as bait, we observed the presence of TakA-Myc (prey). The Co-IP experiment demonstrated that TglT and TakA interact directly with each other. This behavior is atypical for type IVA family members.

TglT preferentially binds GTPn. Like AbiEii, TglT belongs to the DUF1814 superfamily and contains similar conserved motifs (Supplementary Fig. 1), suggesting that it may bind NTP. We mixed purified TglT with [α-32P] labeled GTP, and resolved the mixtures by native-PAGE. A radioautograph of the native-PAGE (Fig. 1e) revealed a predominant band corresponding to the molecular mass of TglT. The intensity of the band increased as the protein concentration increased suggesting that TglT and GTP form a stable complex. The same experiment was performed using [α-32P] labeled ATP, UTP and CTP, respectively, however we did not observe the ATP-TglT, UTP-TglT or CTP-TglT complexes (Fig. 1f). To confirm GTP specificity, we performed a competition assay. After the formation of the TglT- [α-32P] GTP complex, we added a large excess of unlabeled ATP, GTP, CTP or UTP. The TglT-[α-32P] GTP complex was broken down only by unlabeled GTP (Fig. 1g). We conclude that TglT specifically binds GTP.

Structural features of TglT. TglT has a compact fold with dimensions of 59.2 Å × 47.7 Å × 43.1 Å. It encompasses mixed β-sheets and α-helices (Fig. 1h). The expression of the Rv1044-Rv1045 operon in the H37Rv genome (not to scale). The location of the genes is indicated. (b) Toxicity and antitoxicity assay of Rv1044-Rv1045 pair in E. coli. The expression of the pBAD33-c-6His-Rv1045 plasmid encoding TglT-His resulted in cell growth arrest; the toxicity was neutralized when the pET28-n-6His-Rv1044 plasmid encoding His-TakA was co-expressed. See plasmid details in Supplementary Table 2. (c) Growth curves of BL21 cells containing plasmids encoding the toxin (pBAD33-c-6His-Rv1045) and the antitoxin (pET28-n-6His-Rv1044), or the toxin with the empty vector. For all experiments, the bacteria were induced when OD600 reached 0.2, which was set as hour 0. The OD600 was then measured at the indicated time points. Red curve, the toxin was induced (+IPTG) first and the antitoxin was induced (+Ara) 2 h later; green curve, the toxin was induced (+IPTG) but the antitoxin was not induced (−Ara); blue curve, BL21 cells containing pBAD33-c-6His-Rv1045 and the empty pET28a but without induction (−Ara); black curve, BL21 cells containing pBAD33-c-6His-Rv1045 and the empty pET28a with induction (+Ara). Data shown are mean OD600 value ± SD (n = 3). (d) The interaction between TglT and TakA was detected by a Co-IP experiment. NTP binding assays demonstrate that toxin TglT preferentially binds GTP. In the upper two native-PAGEs, TglT forms a complex with radioactively labeled GTP, but not with ATP, CTP or UTP. The concentration of all NTPs in the assays was constant (3.3 nM). The concentrations of TglT and [α-32P] labeled GTP were 8 μM and 3.3 nM, whereas the concentrations of the cold competitors were 0.5 μM, 4 μM and 2 μM. The bottom native-PAGE shows competition binding. The concentrations of TglT and [α-32P] labeled GTP were 8 μM and 3.3 nM, whereas the concentrations of the cold competitors were 0.5 μM, 4 μM and 16 μM, respectively. Source data are provided as a Supplementary Data 2.
group of the substrate. Helices α6 and α7 of CTD harbor the conserved motif 3 and motif 4. They constitute the right-side wall of the central cavity (Fig. 3b). K189 in motif 3, as well as H207 and D208 in motif 4 are located inside the central cavity, suggesting their involvement in catalytic activity. Despite extensive efforts, neither the crystallization of TglT-GTP complex nor the soaking of concentrated GTP with TglT crystals was successful. Therefore, the exact function of these conserved residues in catalysis requires further investigation.

The TglT structure shares limited similarity with known proteins. We compared the TglT structure against all entries in PDB using the Dali server\(^\text{30}\). The best hit was JHP933, a putative nucleotidyltransferase from *H. pylori*, PDB id: 4OK0\(^\text{31}\) (Supplementary Table 1). The alignment gave a Dali Z-score = 14.4 with the rmsd = 4.1 Å and 199 C\(_\alpha\) aligned. JHP933 is another DUF1814 family member, which shares 16% sequence identity with TglT.

The NTase motifs of TglT are important to toxicity. We performed a mutagenesis study to investigate whether the conserved motifs of TglT are important to toxicity. We introduced mutation to each of the five conserved motifs identified in the catalytic center, including motifs 1, 2, 2a in the NTD and motifs 3 and 4 in the CTD (Fig. 3c, d, Supplementary Fig. 1). Whatever possible we targeted invariant and charged residues, because they are more likely playing critical roles in catalysis. We chose G62 because it is the only invariant residue in the motif 1. Motif 2a does not contain invariant residue, however residue 146 is highly conserved and negatively charged. Additionally, we investigated position of selected residues in the structure of TglT to ensure that their side chains have access to the central cavity (Fig. 3b).

While the expression of wildtype TglT inhibited bacterial growth, mutants G62A (motif 1), D82A (motif 2), K189N (motif 3) and D208A (motif 4) were non-toxic (Fig. 3e). Surprisingly, mutant

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**Fig. 2 The crystal structure of TglT reveals a putative catalytic cavity.** a Ribbon model of the TglT crystal structure with annotated secondary structure elements. The phosphorylated S78 between NTD and CTD is highlighted with a stick model. Left, standard view; right, 180° rotation around the vertical axis. b Solvent accessible molecular surface of TglT colored by potential from −15 kT/e (red) to 9 kT/e (blue). The phosphate group (spheres) of SEP78 protrudes out of the left wall of the central cavity. Left, standard view; right, 180° rotation around the vertical axis. c Molecular surface of non-phosphorylated TglT with the same view and coloring scheme as panel b.
Fig. 3 Key structural features and the conserved motifs of TglT. a Ribbon model of TglT NTD with the common NTase core highlighted in color and annotated secondary structures. The conserved motifs 1, 2 and 2a, and the conserved Asp/Glu residues are labeled. b Standar view of TglT structure with highlighted and color-coded conserved motifs 1 (yellow), 2 (brown), 2a (blue), 3 (red) and 4 (magenta). Catalytically important residues from these conserved domains are shown in stick model, and annotated. Two hydrogen bonds stabilizing the phosphate of SEP78 are shown with dashed lines. c Secondary structure diagram, and d 1-D diagram of TglT with detailed structural features; the color code is the same as panel a. e Mutations in conserved motifs affect the toxicity of TglT. Bacteria expressing the annotated proteins were spotted on M9 plates (without and with L-arabinose) in 10-fold serial dilutions, from the right to the left: $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$. Cell growth was examined after overnight incubation. The expression of TglT and its variants is verified three hours post induction by western blotting, left. f A magnified view of the phosphorylation site at the active site of TglT. The composite omit map calculated with simulated annealing is superimposed with the atomic model. Residues around SEP78 are annotated. The phosphate group fit the map, indicated with a black triangle. Source data are provided as a Supplementary Data 2.
E146Q (motif 2a) exhibited toxicity similar to the wildtype protein. We next compared the initial growth rates of E146Q, D82A and wildtype TglT, which revealed a clear difference 4 hours after induction (Supplementary Fig. 2). While the growth rate of D82A exhibited negligible differences from the plasmid control, the growth rate of E146Q was significantly lower. On the contrary, the density of E. coli expressing wildtype TglT started to decline rapidly. The difference in initial growth rates was probably below the detection limit of the growth inhibition assay (Fig. 3c). Thus, E146Q mutation on motif 2a led to attenuated toxicity rather than complete loss of function. Collectively, our findings suggest that all conserved motifs constituting the central cavity are important to the toxicity of TglT.

Crystalllographic study revealed the phosphorylation of TglT. We observed a strong positive difference map peak connected to the hydroxyl side chain of residue S78 of wildtype TglT, which was absent in D82A (Supplementary Fig. 3). The additional electron density could accommodate a phosphate, thus we modeled a phosphoserine (SEP) at this site instead of a serine. The additional electron density of hydrogen side chain of residue S78 of wildtype TglT, which permits phosphorylation and more importantly the phosphorylation of TglT was attributed to TakA.

TglT phosphorylation was attributed to TakA. To confirm S78 phosphorylation, we employed mass spectrometry. Wildtype TglT and the mutant S78A co-expressed with TakA were analyzed with LC-MS/MS. A fragment \( \gamma^7 \text{GIPPSRTSKKDFDTVAR}_{98} \) from wildtype TglT was observed; the calculated molecular mass for S78 was 184,998 Da, matching the theoretical molecular mass of phosphoserine, 185.07 Da, confirming that S78 was phosphorylated (Fig. 4a, Supplementary Fig. 5). The fragment \( \gamma^7 \text{AKDFDTVAR}_{86} \) from S78A mutant was observed; the calculated molecular mass of A78 was 89,037 Da, matching the theoretical molecular mass of alanine, 89.09 Da, confirming that the mutant residue was an alanine (Fig. 4b, Supplementary Fig. 6).

Next, we examined the phosphorylation of a selection of TglT mutants in E. coli using Phos-tag SDS-PAGE. Wildtype TglT co-expressed with TakA showed two bands in the Phos-tag SDS-PAGE, a major upper band and a minor lower band (Fig. 4c). We analyzed the two species directly from the PAGE by LC-MS/MS, confirming that the upper band was the phosphorylated TglT (Supplementary Fig. 7), whereas the lower band was the non-phosphorylated TglT (Supplementary Fig. 8). Mutants S78A, S78D and S78D do not contain the phosphorylation target, and we did not observe the upper band corresponding to the phosphorylated species. G62A (motif 1) accounted for the major loss of phosphorylation, however a minor phosphorylated portion (upper band) was faintly visible. D82A (motif 2), either expressed alone or co-expressed with TakA, exhibited a single band in Phos-tag PAGE. The band was slightly higher than the non-phosphorylated species observed for wildtype protein, but was lower than the phosphorylated band. Our crystallographic study showed that the D82A structure was non-phosphorylated. Collectively, D82A remained non-phosphorylated in the presence of TakA. K189N and D208A from motifs 3 and 4 impaired phosphorylation. The majority of these two mutants remained non-phosphorylated when co-expressed with TakA (Fig. 4c). E146Q (motif 2a) was the only exception as it was highly phosphorylated when co-expressed with TakA. We analyzed the lower and upper bands of E146Q by LC-MS/MS, confirming that while the upper major band was the phosphorylated E146Q, the lower minor band was the non-phosphorylated E146Q (Supplementary Figs. 9 and 10). In summary, with the exception of E146Q, all of the examined mutations within the conserved motifs of TglT impaired the phosphorylation of TglT.

TakA phosphorylates TglT directly. Our results demonstrated that TglT phosphorylation was always coupled with TakA co-expression, which strongly hinted the direct role of TakA in phosphorylation. To confirm this postulation, we devised an in vitro kinase assay using purified TakA (Supplementary Fig. 11A, B) and TglT. When incubating MBP-TakA with TglT (Fig. 5a), we detected radioactively labeled TglT species in SDS-PAGE. The signal vanished when the TglT S78A mutant was used in the reaction (MBP did not affect the kinase assay, Fig. 5a lane 3&7). The result provided the evidence that TakA is directly responsible for the phosphorylation of TglT at the residue S78 in vitro, which demonstrated that the antitoxin TakA is a novel serine protein kinase.

We found it difficult to understand why conserved motifs 1–4 were required for TglT phosphorylation, while E146Q in motif 2a did not cause loss of phosphorylation activity in E. coli (Fig. 4c). Therefore, we used the kinase assay to compare the phosphorylation efficiency of TglT mutant E146Q and wildtype TglT (Fig. 5b). Our results showed that TakA phosphorylated wildtype TglT with higher efficiency than E146Q, whereas TakA did not phosphorylate S78A even at the highest kinase concentration. Thus, motif 2a is also important in TakA catalyzed phosphorylation, although
its role is not as critical as those of the other motifs. The discrepancy between the in vivo and the in vitro results was likely due to various reasons. The phosphorylation of TglT mutant E146Q by TakA in \textit{E. coli} might benefit from the higher local concentration of TakA, longer incubation time and better physiological conditions (overnight \textit{E. coli} culture at 37 °C vs 45 min in vitro incubation at 25°C), which eventually compensated phosphorylation impairment caused by E146Q. Phosphorylation of TglT leads to toxicity inhibition. Our crystallographic studies demonstrated that the presence of SEP78 not only introduced steric hindrance at the catalytic center but also altered the local electrostatic charging, both of which might hinder the substrate entry into the catalytic cavity (Fig.2b, c). Thus, the phosphorylation at S78 may inhibit the activity of TglT, and in turn the toxic function. To test our hypothesis, we first compared the GTP binding affinity of the phosphorylated and non-phosphorylated TglT species (Supplementary Fig. 12). It was evident that the phosphorylated TglT exhibited lower GTP binding affinity than the non-phosphorylated TglT, which supports the theory that SEP78 impairs GTP binding. Next, we assessed the toxicity of TglT mutant S78D (functioning as a mimic of the phosphoserine). As expected, the S78D was non-toxic (Fig. 3e). Finally, we performed the kill-and-rescue experiment. TglT and variants were cloned into L-arabinose inducible expression vector pBAD33, whereas TakA was cloned to an IPTG inducible vector pET28a. Upon induction, the toxic effect of wildtype TglT was rescued by TakA efficiently (Fig.6a). The S78A prevented phosphorylation, thus S78A was toxic and the toxicity was not rescuable. The E146Q mutant exhibited toxicity similar to the wildtype TglT, which was rescued by TakA. However, the rescuing of E146Q toxicity was much less efficient (~10 fold less) than the rescuing the toxicity of the wildtype TglT (Fig. 6a). This observation is consistent with our in vitro kinase assay, in which the TakA catalyzed phosphorylation of the mutant E146Q was less efficient than phosphorylation of the wildtype TglT (Fig. 5b). The phosphorylation of TglT is directly coupled with toxicity neutralization. Collectively, our results support an unusual antitoxicity mechanism, in which the antitoxin TakA is a novel protein kinase that phosphorylates the cognate toxin TglT, thereby inhibiting its toxicity. TakA is a novel atypical kinase. TakA shares limited homology with the structurally characterized proteins, thus, we employed the HHpred server \textsuperscript{34} to detect remote homology and to predict its structure. The best hit is \textit{Rv2827c} (probability score = 99.59, PDB ID: 1ZEL) from \textit{Mtb}. \textit{Rv2827c} contains an N-terminal wHTH domain and a C-terminal uncharacterized domain \textsuperscript{35}. Based on our biochemical characterization and HHpred prediction, we postulated that TakA and \textit{Rv2827c} belong to the same kinase family which encompasses an N-terminal wHTH domain and a C-terminal domain. Thus, the conserved residues identified by sequence alignment might reveal catalytically important residues (Supplementary Fig. 13). To support our theory, we performed site-directed mutagenesis studies. As shown in Fig. 5c, G71, E72 and D74 are non-conserved residues located between wHTH and C-terminal domain. While G71A and D74A led to major loss of kinase activity, E72A retained most of its activity. In contrast, nearly all mutations of the conserved residues S93, H98, P105 and D155 located within C-terminal domain resulted in the abolishment of activity.
Mtbb encodes 11 eukaryotic-like serine/threonine protein kinases (STPKs), from PknA to PknL. Some STPKs are essential for Mtbb growth, while others play an important role in virulence, adaption and survival in animal models or macrophage in vitro; however, none of these STPKs is similar to TakA.

In the search for structural homolog of Rv2827c using Dali server, we found a rather remote homolog, Rio2 (Z-score = 5.5). Rio2 belongs to an atypical serine/threonine kinase family. Rio2 contains an N-terminal PHD domain followed by a C-terminal protein kinase domain, a domain organization shared by Rv2827c and TakA. See Supplementary Data 3 for the Dali search full results.

TakA negatively autoregulates Rv1044-Rv1045 promoter. Given that TakA contains a putative N-terminal PHD domain (Supplementary Fig. 13), it may play a role in promoter regulation. To investigate whether TakA autoregulates its own promoter, we constructed a P_{Rv1044-lacZ} fusion reporter in M. smegmatis mc^{2}-155 (Fig. 7a). Expression of Rv1044 either alone or together with Rv1045 strongly repressed transcription of the P_{Rv1044-lacZ} fusion (Fig. 7b), indicating that TakA negatively autoregulates the Rv1044-Rv1045 promoter.

Discussion
Combining of our mutagenesis and functional studies, we concluded that the active site plays at least two distinct roles. First, it...
is the catalytic center of the putative guanylyltransferase, whose catalytic activity is responsible for the toxicity. We demonstrated that mutations in these conserved motifs led to the loss of toxicity (Fig. 3e, Supplementary Fig. 2), thus linking the active site to the toxic function. Second, the active site of TglT is also the substrate of the kinase TakA. In Phos-tag SDS-PAGE and in vitro kinase assays (Fig. 4c, Fig. 5a, b), we showed that mutations in the conserved motif impaired the kinase reaction catalyzed by TakA. Although phosphorylation of TglT mutant E146Q by TakA exhibited a similar-to-wildtype level in E. coli, the phosphorylation efficiency of purified TglT E146Q in vitro was much lower than wildtype level (Fig. 5b). These results suggest that in order to phosphorylate S78 of TglT, its entire active site is required by kinase TakA. This finding is consistent with our Co-IP experiment, which confirmed the direct interaction between TglT and TakA.

The known antitoxicity mechanisms are fundamentally different from what we report here, which involves phosphorylation of the toxin. It is intriguing that although the phosphorylation target S78 is located at the very center of the catalytic cavity and is highly conserved, this residue is nonessential for toxin function (Fig. 3e).

Fig. 6 Phosphorylation of TglT results in toxicity neutralization and a proposed model of the Rv1044-Rv1045 TA system. a The antitoxicity mechanism of TakA involves phosphorylation of TglT at S78 site. BL21 cells expressing annotated proteins were spotted on M9 plates with 10-fold serial dilutions from the right to the left: 10⁻¹ 10⁻² 10⁻³, 10⁻⁴, 10⁻⁵ and 10⁻⁶, respectively. The plate on the left contained IPTG inducing TakA expression, whereas the plate on the right contained both IPTG and L-arabinose inducing toxin and antitoxin expressions. Bacterial growth was examined after overnight incubation. The toxicity of S78A was not rescued by TakA because the mutant cannot be phosphorylated. The neutralization of TglT E146Q toxicity was about 10-fold less efficient than that of wildtype TglT, because the phosphorylation of TglT E146Q by TakA was also less efficient (Fig. 5b). The Rv1044 gene (blue arrow) encodes the atypical serine protein kinase TakA (blue ellipse), acting as the antitoxin, while the Rv1045 gene (orange arrow) encodes the guanylyltransferase TglT (orange rectangle), acting as the toxin. TakA negatively regulates its own promoter. TglT binds GTP and targets a vital cellular process, which leads to bacterial growth arrest. A possible cellular process targeted by TglT is protein translation. TakA recognizes TglT and phosphorylates the S78 residue, thus inhibiting the catalytic activity of TglT resulting in the neutralization of toxicity. The gray dashed arrow indicates the reversal of antitoxicity, which required an unknown phosphatase. The PstP phosphatase encoded by Mtb is a possible candidate. Source data are provided as a Supplementary Data 2.
tested its ability to dephosphorylate TglT. As shown in Supplementary Fig. 14A, PstP (0.4–2 μM) could completely dephosphorylate PknB (5 μM) in 30 minutes, indicating a phosphatase activity similar to that reported by Biotel et al.41. By contrast, we observed only slight increase in the dephosphorylated TglT species in the gel. To confirm the above observations, we extended the incubation time of the phosphatase assays to 2 h, 4 h and 12 h, respectively. While most of TglT still remained unchanged in the presence of MBP-TakA (Supplementary Fig. 14B), it became evident that PstP could indeed dephosphorylate TglT (Supplementary Fig. 14C), although in a rather unspecific manner. PstP required 12 h to fully dephosphorylate TglT, which was a slower rate than was observed for the dephosphorylation of its own substrate PknB. Taken together, these results suggest a possible role of PstP phosphatase in TglT dephosphorylation. Nevertheless, whether PstP phosphatase is able to reverse the TglT phosphorylation and finally leads to the activation of the TA system remains to be verified in vivo. Another challenging study is the existence of other unknown Ser/Thr phosphatases in Mtb that can specifically and efficiently dephosphorylate TglT. The solution of the above scientific questions is the focus of our ongoing studies, and the exact mechanism of TglT activation will be finally clarified.

To understand the potential cellular processes targeted by TglT, we employed RNA sequencing (RNA-seq) to study the transcriptomic profiles of bacteria expressing wildtype TglT and the nontoxic TglT mutant D82A. Sequencing libraries were constructed and sequenced, which resulted in the identification of 2604 differentially expressed genes (DEGs) between the bacteria expressing wildtype TglT and the D82A mutant (Supplementary Fig. 15A). Among these DEGs, there are 2078 up and 526 downregulated genes (Supplementary Fig. 15B, C and Supplementary dataset 1). Gene Ontology (GO) enrichment analysis of the DEGs showed that the toxin TglT induced gene expression changes were preferentially correlated with ribosome-related genes (Supplementary Fig. 15D). KEGG pathway enrichment analysis implied that TglT mostly alters ribosome and metabolic pathways (Supplementary Fig. 15E). Together, these analyses suggest that TglT most likely targets genes involved in protein translation. Notwithstanding, the RNA-seq is an indirect method to investigate the cellular process targeted by TglT. Assessing the protein translation level in bacteria overexpressing the toxin is an important experiment to understand whether the TglT inhibits translation in vivo. To identify the substrate of TglT via in vitro translation assay and mass spectrometry is our current goal, which will eventually reveal the enzymatic characteristics of this novel NTase.

In summary, our results suggest a model for the TA system Rv1044-Rv1045 from Mtb (Fig. 6b). Rv1044 encodes the atypical serine protein kinase TakA acting as the antitoxin, and Rv1045 encodes the guanylyltransferase TglT acting as the toxin. The promoter of the Rv1044-Rv1045 TA is negatively autoregulated by TakA. The catalytic activity of TglT requires GTP, which leads to toxicity and eventually bacterial growth arrest. Protein translation is a possible cellular process targeted by TglT. Assessing the protein translation level in bacteria overexpressing the toxin is an important experiment to understand whether the TglT inhibits translation in vivo. To identify the substrate of TglT via in vitro translation assay and mass spectrometry is our current goal, which will eventually reveal the enzymatic characteristics of this novel NTase.
families COG5340 and DUF1814, which are widespread in prokaryotes.

Methods

Reagents. All chemicals used in this study were purchased from Sigma-Aldrich unless otherwise specified.

Bacterial strains and culture. Bacteria were grown at 37 °C in LB medium or M9 minimal media supplemented with 0.2% casamino acids. Antibiotics used were 100 μg/ml ampicillin, 50 μg/ml kanamycin and 25 μg/ml chloramphenicol, as indicated. Expression of the recombinant proteins was induced by IPTG or IPTG.

Bioinformatic analyses. Structural-based multi-sequence alignment was performed using programs MUSCLE24 and HHpred34. Program ESPript43 was used for generating multi-sequence alignment figures. Phosphorylation site prediction was conducted using NetPhos 3.1 server25.

Plasmid construction. All plasmids used in this study are listed in Supplementary Table 2. The genes of toxin and antitoxin Rv1045 and Rv1046 were amplified from MTB H37Rv strain genomic DNA by PCR. The genomic DNA was a generous gift from Hairong Huang’s lab, Beijing chest hospital, Capital Medical University. Plasmids used for expression in this study include pET28a (+) (Novagen), pBAD/Myc-His-A (Thermo Fisher Scientific), pBAD3X, pMAL-c5x (NEW ENGLAND BioLabs) and pETDuet-1 (Novagen).

Constructing plasmids for E. coli toxicity assays, Rv1045 gene was amplified by PCR, treated with restriction enzymes KpnI and HindIII, and was subsequently inserted into pBAD33, encoding wildtype TakA, to yield a C-terminal 6×His-tag.

Constructing a plasmid for toxin neutralization, Rv1046 gene was amplified by PCR, treated with NdeI and Xhol enzymes and was inserted into pET28a, encoding antitoxin TakA with a N-terminal 6×His-tag.

Constructing plasmid for Co-IPs, Rv1045 gene was amplified by PCR, the coding region for the FLAG-tag, DYKDDDDK, was introduced to the C-terminus of the protein via 3′ primer. The DNA fragment was cloned into pET28a between NcoI and XhoII restriction sites. Rv1046 gene was amplified by PCR and cloned between NcoI and HindIII sites of pBAD/Myc-His-A, yielding the C-Myc-tag plus 6×His-tag protein.

For MBP tagged TakA expression, Rv1045 gene was amplified by PCR; the fragment was cloned between NcoI and HindIII restriction sites of pMAL-c5x, yielding N-terminal MBP tagged TakA.

For crystallization of TgT and variants, Rv1045 gene was cloned between NdeI and XhoII restriction sites of pET28a (+), yielding N-terminal His-tagged protein. Rv1044 gene was cloned between restriction sites Ncol and HindIII of MCS-1 of pETDuet-1, yielding non-tagged protein. TgT and variants were expressed alone or co-expressed with TakA depending on the purpose of the experiments, reducing the toxicity of obtaining phosphorylated proteins.

For expressing non-phosphorylated wildtype TgT protein, pET28a-n-his- Rv1045 was co-transformed with pMAL-c5x empty plasmid, yielding minimal quantity sufficient for biochemical characterizations.

All plasmids encoding protein mutants were constructed using site-directed mutagenesis (QuickChange). All plasmids were verified by DNA sequencing.

Protein expression and purification. The plasmids encoding TgT and variants were transformed to E. coli BL21 (DE3) competent cells. The bacteria culture was grown in LB medium at 37 °C. The induction was initiated by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to 0.5 mM when the culture reached a density of OD600 = 1.0. The culture was cooled to 18°C and the shaking continued over-night. Bacteria were harvested and re-suspended in the lysis buffer and disrupted by ultrasonication. The supernatant was washed with a washing buffer containing 50 mM Tris pH = 8.0, 150 mM NaCl. The protein was eluted using an elution buffer containing 50 mM Tris, pH = 8.0, 150 mM NaCl and 10 mM maltose. The eluate was loaded to HiTrap S HP column (GE Healthcare) and eluted with the linear gradient of 75–1000 mM NaCl.

The eluted protein was finally purified with Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with 20 mM Tris- HCl pH = 8.0, 100 mM NaCl.

Co-immunoprecipitation (Co-IP). Rv1045 and Rv1046 genes were amplified by PCR and cloned to pET28a and pBAD/Myc-His-A vectors, between the restriction sites Ncol/Xhol and NcoI/HindIII, respectively. Plasmid pET28a-c-FLAG-Rv1045 encodes TgT with C-terminal FLAG-tag (termed TgT-FLAG), whereas plasmid pBAD/Myc-Rv1044 encodes a C-terminal Myc tag and C-terminal His tagged TakA (termed TakA-Myc-His). The plasmids were transformed to BL21 DE3 competent cells. A single colony was picked to inoculate 2 ml of LB medium and incubated overnight at 37 °C. Then, the cells were diluted by 10-fold in 20 ml fresh LB medium. The cultures were grown at 37 °C till OD600 = 0.4. The expression of TgT and TakA-Myc-His was induced by the addition of 0.5 mM IPTG and 0.2% (v/v) IPTG.

Bacterial strains and culture. Bacteria were grown at 37 °C in LB medium or M9 minimal media supplemented with 0.2% casamino acids. Antibiotics used were 100 μg/ml ampicillin, 50 μg/ml kanamycin and 25 μg/ml chloramphenicol, as indicated. Expression of the recombinant proteins was induced by IPTG or IPTG.

Western blot. Tagged proteins (His, Myc or FLAG epitope), separated by SDS-PAGE, were transferred to a PVDF membrane (Bio-Rad) using a Criterion blotter (Bio-Rad). The membrane was washed with Western wash buffer (TBS + 0.1% Tween 20) and blocked for 1 hour with 5% skim milk powder at room temperature.

Protein phosphatase assay. The protein phosphatase assay was performed as previously described with minor modifications. Recombinant PptP27 and PknH1-279aa proteins were expressed and purified following the methods published previously. The reaction mixture contained 20 mM Tris-HCl pH = 8.0, 100 mM NaCl, 1 mM DTT, 2 mM MnCl2 and 5 μM protein substrate (TgT or PknB279aa). The reaction was initiated by adding MBP-Taka or PstP phosphatase (concentrations, 0.4 μM) at 37 °C for 60 min. The reactions were incubated at 35 °C. The reaction products were finally loaded onto Phos-tag SDS-PAGE (Wako Pure Chemical Industries) and stained with Coomassie R250.

Protein phosphatase assay. The protein phosphatase assay was performed as previously described with minor modifications. Recombinant PptP27 and PknH1-279aa proteins were expressed and purified following the methods published previously. The reaction mixture contained 20 mM Tris-HCl pH = 8.0, 100 mM NaCl, 1 mM DTT, 2 mM MnCl2 and 5 μM protein substrate (TgT or PknB279aa). The reaction was initiated by adding MBP-Taka or PstP phosphatase (concentrations, 0.4 μM) at 37 °C for 60 min. The reactions were incubated at 35 °C. The reaction products were finally loaded onto Phos-tag SDS-PAGE (Wako Pure Chemical Industries) and stained with Coomassie R250.
Crystallography and structure determination. To gain structural insights into Rv1044-Rv1045 TA we carried out crystallographic studies. Due to the intrinsic toxicity of the wildtype TA, completely arrested E. coli growth and eventually led to cell lysis after induction overnight. We therefore devised two strategies: (a) Introduction of mutations to the conserved motifs, which abolished or attenuated the toxicity, resulting in mutants that were expressible. These conserved motifs include motif I & II from the catalytic motifs of the DNA polI family and motifs III & IV exclusive to the DUF1814 family26. (b) Co-expression with the antitoxin TakA, which neutralizes the toxicity of TgtT, and allows the expression of the wildtype toxin.

The toxin TgtT was crystallized in a hanging-drop vapor-diffusion system at 22°C. The protein was concentrated to 8 mg/ml. The crystallization was carried out by mixing 1 μl of protein sample with 1 μl of reservoir buffer containing 0.1 M Bis-Tris pH = 6.5, 0.2 M magnesium chloride hexahydrate and 25% polyethylene glycol 3350 (v/v). The crystals of TgtT appeared after 5 days; they exhibited a rod-like shape with an average size of 0.3–0.5 millimeters. The cryocooling of the crystals was performed by soaking the crystals in the reservoir buffer containing 10% ethylene glycol for 30 minutes before being frozen in liquid nitrogen.

There are no known TgtT homologous structures for molecular replacement, however protein sequence contains four internal methionine residues, which allowed exploiting the single-wavelength anomalous diffraction method for phasing. Redundant X-ray diffraction data for TgtT mutant D28A crystal containing selenomethionine were collected at beamline BL19U of Shanghai synchrotron radiation facility (SSRF). The X-ray source had a wavelength of 0.979 Å. At the peak adaption edge of the Se atoms. D28A crystals belonged to the space group P321 and diffracted the X-ray to 1.9 Å. The data was processed using the XDS package24. Single copy of TgtT was found in an asymmetric unit, and significant anomalous differences correlation was observed up to 2.8 Å. Software AUTOSHARP/SHARP25 was used to locate 4 Se atoms and to calculate the initial phases, yielding an interpretable electron density map. A preliminary atomic model of D28A was built automatically by ARP/WARP26. The model was then completed by manual building using the Coot27, and it was finally refined using the PHENIX software28. In the final model, we located 290 out of total 293 residues of TgtT, including 4 selenomethionine. The X-ray diffraction datasets for the wildtype TgtT crystals and other mutants reported in this paper were collected in SSRF and at PX III beamline at the Swiss Light Source, Paul Scherrer Institute (Villigen Switzerland). Wavelength used for data collection were 0.98 Å. All structures were solved by molecular replacement with Phaser program29 using D28A as the search model.

In the finally refined the structures, the Ramachandran statistics are: TgtT D28A (alone expressed, Se-Met crystal), 96.88% favored, 2.77% allowed and 0.35% outliers; TgtT wildtype (co-expressed with TakA), 96.83% favored, 3.17% allowed and zero outliers; TgtT S78A (alone expressed), 97.22% favored, 2.43% allowed and 0.35% outliers; TgtT E146Q (alone expressed), 96.18% favored, 3.82% allowed and zero outliers; TgtT S78A (co-expressed with TakA), 96.86%, 2.79% and 0.35% outliers; TgtT D28A (co-expressed with TakA), 96.53% favored, 3.47% allowed and zero outliers; TgtT E146Q (co-expressed with TakA), 96.10% favored, 3.90% allowed and zero outliers.

The statistics of data collection, reduction and structure refinement are summarized in Tables 1 and 2. The final 2Fo-FC map of the crystal structure of wildtype TgtT is shown in Supplementary Fig. 17.

Toxicity and antitoxicity assay. Rv1045 and Rv1044 genes were amplified and cloned into pBAD33 and pET28a vectors respectively, encoding TgtT-His and His-TakA (Supplementary Table 2). The plasmids were transformed to BL21 (DE3) competent cells. Single colonies were isolated and inoculated in 2 ml LB medium overnight. The culture was used to inoculate (1:100) 20 ml of M9 medium; the cultures were grown to OD600 = 0.8. The bacteria were then streaked on M9 plate supplemented with inducer IPTG (16 μM) or i-arabinose (0.2% w/v); the plates were incubated overnight incubation at 37 °C before the examination of cell growth.

Kill-and-rescue assay. Compatible plasmid pairs (pBAD33 and pET28a with and without Rv1044 and Rv1045 genes) were co-transformed to BL21 (DE3) competent cells. The overnight culture was diluted 100 folds in 20 ml fresh M9 minimal medium containing 25 μg/ml chloramphenicol and 50 μg/ml kanamycin, and the diluted cultures were grown to a density OD600 = 0.8 at 37 °C. The cultures were then further diluted by 10-fold serial dilution, from 10−1 to 10−6. Each dilution was finally spotted (1 μl) onto M9 agar plates containing chloramphenicol, kanamycin and i-arabinose. 0.2% i-arabinose and 16 μM IPTG. The plates were incubated overnight at 37 °C before bacterial growth was examined.

NTP binding assay. The purified His-TgtT protein at various concentration was incubated with 3.3 nM (final concentration) of [α-32P]NTPs in a binding buffer containing 50 mM Tris-HCl, 1.5 mM MgCl2, 100 mM NaCl, pH = 8.0 at 37 °C for 1 h. The resulting mixtures were then resolved by 10% Tris-Glycine native-PAGE running on ice for 70 min at 100 volts (constant). The gels were exposed overnight to a phosphor screen and analyzed using GE TyphoonFLA FLA 7000 biomolecular imager. To investigate GTP binding capacity of TgtT, a concentration series of TgtT were prepared. The highest concentration was 16 μM, followed by 13 steps of two-fold serial dilutions, resulting the following concentrations: 8 μM, 4 μM, 2 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM, 0.0625 μM, 0.0313 μM, 0.0156 μM, 0.0078 μM, 0.0039 μM and 0.002 μM. Only the top four concentrations (16 μM, 8 μM, 4 μM and 2 μM) were used for studying binding with the other NTPs. In the competition binding experiments, 8 μM TgtT protein was pre-incubated with 3.3 μM of radioactively labeled GTP and the unlabeled NTPs were then added to the mixtures as competitors, reaching the final concentrations of 0.5 μM, 4 μM and 16 μM, respectively.

In vitro kinase assay. The kinase reaction mixture (20 μl) contained 10 mM Tris-HCI pH = 8.0, 75 mM NaCl, 10 mM MgCl2, and 50 mM MnCl2, 2.5 mM γ-[32P]ATP, 150 μM unlabeled ATP and 50 μM of protein substrate (TgtT or variants). The reaction was initiated by adding 5 μM MBP-TakA kinase and the incubation time was set at 15 min at 37 °C. The samples were boiled in a 1:10 ratio and run on a 15% SDS-PAGE. The electrophoresis started with a constant voltage of 180 V for 40 min. To analyze the results, the SDS-PAGE was first exposed to a phosphor screen and visualized by GE TyphoonFLA FLA 7000 biomolecular imager. The same SDS-PAGE was then stained with Coomassie Brilliant Blue R250 to visualize all proteins.

Promoter activity assay. The lacZ reporter gene was first amplified using plasmid pMV261-null-lacZ30 as PCR template, and then inserted into the chromosome of M. smegmatis mc2-155 to replace MSMEG_1243_1248 genes using the method described previously31. Next, a DNA fragment containing a 460 bp homology sequence of MSMEG_1242 gene, a T432 terminator (32 bp), a 500 bp upstream of Rv1044 gene (Supplementary Table 3) and a 501 bp homology sequence of lacZ gene were synthesized and inserted into the above strain using the methods described previously32, resulting in the M. smegmatis MSMEG_1242-lacZ reporter strain (Fig. 7a). To construct the plasmids for expression of Rv1044 and Rv1044-Rv1045, their encoding genes were amplified from Mtb genomic DNA by PCR and were subcloned into a shuttle vector pYC601 containing anahydrotetracycline (ATC)-inducible promoter using Seamless Cloning and Assembly Kit. The resulting plasmids and the empty vector were further transformed into the M. smegmatis MSMEG_1242-lacZ reporter strain and then used for Rv1044 promoter activity analysis. 100 ng/ml ATC was added to induce the expression of the antitoxin and the toxin-antitoxin complex. β-galactosidase promoter activity assays were performed according to the standard Miller method33.

RNA sequencing (RNA-seq). Plasmids encoding wildtype TgtT and mutant D28A were transformed to BL21 (DE3) competent cells. The bacteria were grown in liquid LB medium containing chloramphenicol (25 μg/ml) at 37 °C to OD600 ≤ 0.6. The cultures were diluted to OD600 = 0.2 before induction by adding 0.2% L-arabinose. 0.5 μl of bacteria cultures was removed after 2 hours induction at 37 °C and quickly frozen with liquid nitrogen for subsequent RNA-Seq (each with three replicates). Our bacteria growth curve showed that toxicity of TgtT manifested about 2 h after induction. Total RNA was extracted from each sample and RNA-Seq was performed using RiboSEQ-500 sequencing system34. The sequence reads were mapped to the reference genome E.coli BL21 using HISAT and Bowtie2 software35,36. Gene expression level for each sample was quantified and normalized utilizing the software package RSEM tool37,38. Differentially expressed genes (DEGs) between bacteria expressing wildtype TgtT and mutant D28A were analyzed using DEGseq method. The statistics were then analyzed by a fold change ≥ 2 and adjusted P-value ≤ 0.00138. Gene Ontology (GO) enrichment analysis39 and KEGG pathway enrichment analysis40 of DEGs were conducted based on Gene Ontology Consortium[http://geneontology.org] and KEGG database (https://www.kegg.jp/kegg/pathway.html).

Statistics and reproducibility. All experiments were performed in independent biological triplicate and the results of replicates were consistent. Statistical analysis was performed using ANOVA analysis with GraphPad Prism 7 (GraphPad, CA, USA). Details of the number of biological replicates are described in the figure legends and Methods. Error bars represent standard deviation. P value of <0.0001 was considered as extremely significant, which is indicated with ***.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
| Data collection and refinement statistics 1. | TgIT D82A alone expressed Se-Met TgIT WT co-expressed with TakA TgIT S78A alone expressed TgIT E146Q alone expressed | (PDB ID: 6J7T) (PDB ID: 6J7S) (PDB ID: 6J7Q) (PDB ID: 6J7O) |
|---|---|---|---|
| **Data collection** | | | |
| **Space group** | P3_21 | P3_21 | P3_21 | P3_21 |
| **Cell dimensions** | | | | |
| a, b, c (Å) | 94.69, 94.69, 61.84 | 95.89, 95.89, 68.89 | 95.41, 95.41, 68.13 | 95.44, 95.44, 69.21 |
| α, β, γ (°) | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 |
| **Resolution (Å)** | 49.37 (1.86) | 28.56 (2.10) | 0.176 (1.33) | 0.081 (0.746) |
| **R_{sym}** | 0.194 (1.65) | 23.47 (3.18) | 97.3 (83.4) | 99.9 (99.9) |
| **Completeness (%)** | 11.04 (11.23) | 99.9 (99.5) | 18.67 (12.46) | 9.97 (9.98) |
| **Re-refinement** | | | | |
| **Resolution (Å)** | 49.37 (1.90) | 28.56 (2.10) | 0.1883/0.2057 (0.4065/0.4211) | 0.1994/0.2311 |
| **No. reflections** | 48568 | 21589 | 0.2463/0.2872 | 0.3017/0.3345 |
| **R_{work}/R_{free}** | 0.176 (1.33) | 0.081 (0.746) | 0.056 (0.961) | 0.078 (1.12) |
| **I/σ(I)** | 14.94 (1.65) | 23.47 (3.18) | 22.42 (2.45) | 17.42 (2.01) |
| **No. atoms** | 2251 | 2214 | 2233 | 2228 |
| **B-factors** | 33.03 | 41.92 | 48.07 | 49.07 |
| **Protein** | 0 | 0 | 0 | 0 |
| **Ligand/ion** | 196 | 204 | 217 | 196 |
| **Water** | 53 | 21 | 0 | 0 |
| **R.m.s. deviations** | | | | |
| **Bond lengths (Å)** | 0.003 | 0.004 | 0.009 | 0.007 |
| **Bond angles (°)** | 0.706 | 0.731 | 0.843 | 0.924 |

*Values in parentheses are for highest-resolution shell.

| Data collection and refinement statistics 2. | TgIT S78A co-expressed with TakA TgIT D82A co-expressed with TakA TgIT E146Q co-expressed with TakA | (PDB ID: 6J7R) (PDB ID: 6J7N) (PDB ID: 6J7P) |
|---|---|---|---|
| **Data collection** | | | |
| **Space group** | P3_21 | P3_21 | P3_21 |
| **Cell dimensions** | | | | |
| a, b, c (Å) | 95.65, 95.65, 68.65 | 95.50, 95.50, 68.18 | 94.69, 94.69, 67.25 |
| α, β, γ (°) | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 | 90.00, 90.00, 120.00 |
| **Resolution (Å)** | 47.82 (2.30) | 47.75 (2.29) | 47.34 (2.63) |
| **R_{sym}** | 0.075 (0.520) | 0.078 (0.906) | 0.149 (0.744) |
| **I/σ(I)** | 3.815 (2.52) | 7.25 (0.87) | 4.45 (0.94) |
| **No. atoms** | 2213 | 2232 | 2219 |
| **B-factors** | 57.81 | 77.97 | 67.04 |
| **Protein** | 0 | 0 | 10 |
| **Ligand/ion** | 0 | 0 | 0 |
| **Water** | 53 | 21 | 10 |
| **R.m.s. deviations** | | | | |
| **Bond lengths (Å)** | 0.010 | 0.007 | 0.004 |
| **Bond angles (°)** | 0.993 | 0.832 | 0.598 |

*Values in parentheses are for highest-resolution shell.
Data availability

All data relevant to this study are supplied in the manuscript and supplementary files or are available from the corresponding author upon request. Coordinates and structure factors are deposited in the Protein Data Bank with the PDB entries: 6T7J, 6T7S, 6T7Q, 6T70, 6T7R, 6T7N and 6TP7. RNA-seq data are deposited in NCBI Sequence Read Archive with accession No. PRJNA560278. The original data underlying Figs. 1b-g, 3c, 4a-c, 6a, 7b and Supplementary Figs. 1, 11, 12, 14 is provided in the Supplementary Data 2. The RNA-seq data underlying Supplementary Fig. 15 is provided in the Supplementary Data 1.

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Author contributions
S.C., S.Y. and X.G. designed the study. S.C., W.J. and X.G. wrote the paper. Y.X., X.G., Z.K., Y.H. and M.X. performed experiments. S.C., S.Y., X.G., W.J., B.Q., H.H and W.M analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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