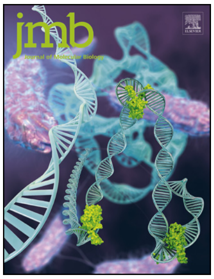

Acetylation of Response Regulator Proteins, TcrX and MtrA in *M. tuberculosis* Tunes their Phosphotransfer Ability and Modulates Two-Component Signaling Crosstalk

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Abstract

Two-component signal transduction (TCS) cascades involve stimulus-dependent activation and phosphorylation of a sensor kinase (SK), which then transfers the phosphoryl moiety to the response regulator (RR) protein. The fidelity of this phosphotransfer reaction from the SK to the RR provides specificity to TCS signaling. In the present study, we show that for TcrX, a transcriptionally autoregulated RR of *Mycobacterium tuberculosis*, acetylation enhances its net phosphorylation from cognate SK TcrY and lowers it from a non-cognate SK MtrB. Similar acetylation mediated increase in phosphorylation was also observed for another RR MtrA from cognate SK MtrB. Thus, we establish a novel TCS signaling design wherein acetylation of RRs results in enhanced cognate phosphorylation and suppresses non-cognate phosphorylation. Using wild-type or acetylation-deficient TcrX proteins in *M. tuberculosis* H37Ra, we demonstrate that non-acetylated TcrX acts as a "phosphate sink" for MtrB and suppressing signal propagation from MtrB to MtrA *in vivo*, linking metabolism to TCS signaling. Overall, we report that acetylation of RRs shields TCSs from crosstalk, modulates the phosphatase activities and alters the DNA-binding activities of RRs, all of which are non-intuitive behavior of TCS systems.

Introduction

Two-component signal transduction (TCS) is one of the basic forms of signaling apparatus present in the bacterial cells, through which they sense their environmental and intracellular cues and mount appropriate adaptive responses. In a typical TCS, environmental cues activate a membrane-bound sensor kinase (SK) protein, which undergoes autophosphorylation on a conserved His residue. The phosphoryl group is then transferred to a conserved Asp residue on the second component, a cytosolic response regulator protein (RR). This alters the activity of the RR toward its target molecule, generally DNA, thereby modulating its activity as a transcription factor [1,2]. Due to the high degree of conservation among the phosphorylation domains among SK and RRs, it is expected that TCSs will demonstrate extensive crosstalk between non-cognate SK–RR pairs [3]. However, such crosstalk has not been observed, leading to the proposal of several underlying processes which govern the specificity of interaction between cognate SK and RR [3–6].

The genome of *Mycobacterium tuberculosis*, the causative bacterium of the disease tuberculosis, encodes 11 paired TCS and a few orphan SKs and

RR proteins [7]. Most TCSs encoded in the genome of *M. tuberculosis* have been shown to be biochemically active, and almost all of them have been linked to the virulence of the tubercle bacilli [8–11]. Recently, it was also shown that the SK MtrB sensor kinase can transfer its phosphoryl moiety to other RRs besides its cognate RR and serves as a promiscuous phosphodonor [8]. Similarly, the RR TcrX was shown to be capable of accepting phosphoryl moiety from more than one SK besides its cognate SK and served as promiscuous

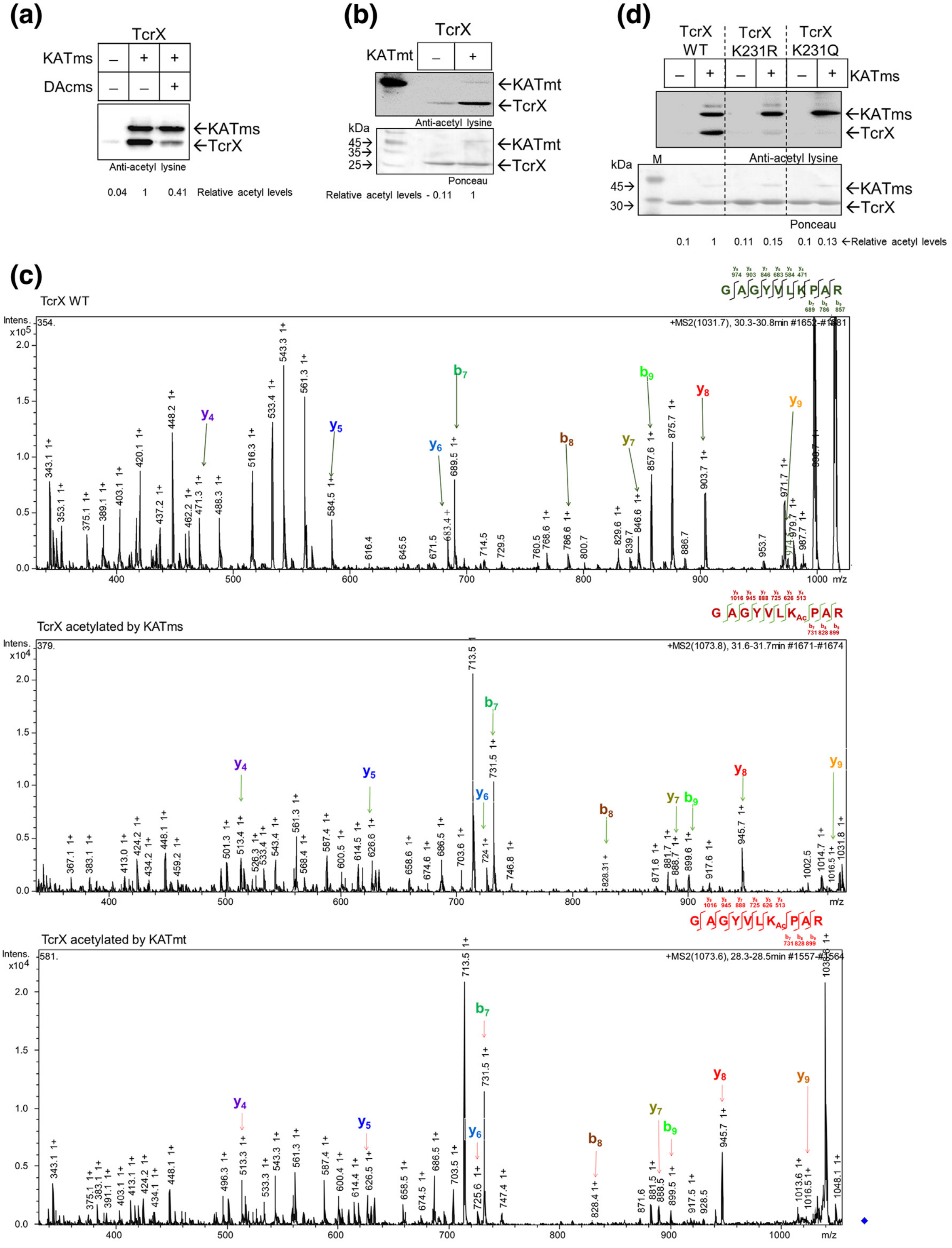


Fig. 1 (*legend on next page*)

acceptor [8]. This report was the first to show significant crosstalk among TCSs (more than 50%) when the predominant view was that of specificity with only 3% crosstalk, as recorded in *Escherichia coli* [12]. Both these proteins turned out to be compromised for specificity [8]; however, it was not clear as to what governs the crosstalk in these mycobacterial proteins, unlike specific signaling dictated by SK–RR interfacial residues [5,13,14] identified in TCS proteins from *E. coli* [12] or *Caulobacter* sp. [15]. While crosstalk helped explain many unusual observations in the area of mycobacterial TCS signaling, including its possible impact on virulence and pathogenesis of the tubercle bacilli, it was mostly based on *in vitro* observations [8,16], warranting further investigation as to its relevance *in vivo* where this crosstalk might be realized.

Addition and removal of acetyl group have been proposed as one of the most effective reversible post-translational modifications, which can modulate protein activities and is comparable to phosphorylation [17–19]. Given that the intracellular levels of acetyl-CoA, acetyl phosphate or acetate are linked to the metabolic status of cells, acetylation of signaling proteins provides a unique mechanism for regulating cellular adaptation through changes in carbon metabolism and intracellular energy levels [20–22]. Acetylation of RRs CheY, RcsB in *E. coli*, PhoP in *S*. Typhimurium and more recently for DosR of *M. tuberculosis* has been shown to change either their phosphorylation or their DNA-binding properties or both [19,23–27]. In *Mycobacterium* spp., protein acetylation has been shown to modulate activities of enzymes involved in fatty acid and propionate metabolism (e.g., FadD), phosphatase PtpB, nucleoid-associated protein HU, and Ku, a DNA repair protein [28–31]. Recently, using mass spectrometry-based acetylome analysis, a number of TCSs proteins were reported to be acetylated in *M. tuberculosis*, including RRs such as RegX3, PhoP, PdtaR, MtrA and TcrX [26,29,32]. However, except for RR DosR [27], no physiological relevance or analysis of these acetylated RRs has been reported till date.

Here we study the impact of acetylation on the functional activities of two RRs, specifically their crosstalk abilities [8]. Toward this two representative TCSs, which participate in crosstalk, MtrAB and TcrXY were selected [8]. The TcrXY system is particularly interesting on the account of its role in virulence of tubercle bacilli and induction of its expression under iron insufficiency [10,33,34]. Similarly, for MtrAB TCS, the RR MtrA has been shown to be essential [35–37], while SK MtrB is dispensable, but regulates cell division [35,37–40]. We demonstrate that site-specific acetylation of the selected RRs alters their phosphorylation potential by both cognate and non-cognate SKs and affects the TCSs signaling landscape. Our results provide evidence of how crosstalk could be generated and regulated inside the cell. Overall, we propose a scheme of TCS signaling where specificity or crosstalk among TCSs is dictated by acetylation status of the participating RRs, thereby linking metabolism with the fidelity of signal transduction.

Results

The major aim of this study was to identify and probe the impact of RR acetylation on inherent phosphorylation potential and associated crosstalk among TCSs of *M. tuberculosis* if any. From the previously reported TCS crosstalk landscape in *M. tuberculosis* [8], we selected TCS TcrXY, wherein RR TcrX is the most promiscuous phospho-acceptor protein, while its cognate SK TcrY specifically transfers phosphate only to TcrX. We selected another TCS MtrAB, where SK MtrB is highly promiscuous, while RR MtrA is extremely specific [3]. The RRs of these two systems hence became very attractive tools to study the impact of acetylation on phosphotransfer specificity determination in TCS signaling.

RR TcrX is acetylated by mycobacterial acetyl transferase

TcrX has been reported to be acetylated in mycobacterial cells [32], and we confirmed this using cAMP-dependent acetyl transferase from both *Mycobacterium smegmatis* KATms (MSMEG_5458)

Fig. 1. Identification and characterization of acetylation of TcrX protein. (a) Acetylation and deacetylation of TcrX protein using acetyltransferase (KATms, MSMEG_5458) and deacetylase (DAcms, MSMEG_5175) enzymes from *M. smegmatis* RR TcrX. Acetylation was probed by Western blotting. (KATms, acetyltransferase; DAcms, deacetylase from *M. smegmatis*). Numbers indicate the quantitative measurement of acetylation on the respective proteins, normalized to acetylated TcrX as 1 (n = 3). (b) Acetylation of TcrX using KATmt (Rv0998) under *in vitro* conditions, identified by Western blotting for acetylated lysine. Top, an image of blot probed for acetylated lysine and bottom, Ponceau stained blot. Numbers indicate the quantitative measurement of acetylation on the respective proteins, normalized to acetylated TcrX as 1 (n = 3). (c) MS/MS analysis for identification of acetylation site on TcrX by KATms (MSMEG_5458). Top panel shows the spectra from wild-type non-acetylated protein and middle, from TcrX acetylated by KATms, and bottom, from TcrX acetylated by KATmt. The ions are colored to facilitated ease of comparison. (d) Acetylation site confirmation in TcrX by site-directed mutagenesis. The wild-type or mutant K231R/K231Q TcrX protein was probed for acetylation from KATms as described in panel b. Top, an image of blot probed for acetylated lysine and bottom, Ponceau stained blot (M, marker). Numbers indicate the quantitative measurement of acetylation on the respective TcrX protein. Amount of signal recorded for acetylated TcrX was taken as 1, and the rest were normalized with respect to that (n = 3).

and *M. tuberculosis* KATmt (Rv0998) using acetyl-CoA as the donor molecule (Fig. 1a and b, respectively). Concurrently, we confirmed that the acetylation was reversible using the deacetylase enzyme DAcms (MSMEG_5175) [28], and distinct deacetylation was also recorded (Fig. 1a, last lane). Considering higher activity and stability for KATms [41], it was used for all subsequent studies.

Next, the lysine residue where acetylation occurs in the TcrX protein was identified using ESI-TOF MS/MS analysis. Inspection of precursor ion spectra of peptide fragments displayed acetylated peptide fragment (537.3^{2+}) in TcrX protein in the presence of acetyltransferases, which was absent in non-acetylated TcrX (516.3^{2+}) (Fig. S1a–c). Interestingly, we identified a unique and hitherto unreported [32] Lys^{231} residue on the peptide "GAGYVLKPAR," in the C-terminal region of the TcrX protein, which was acetylated by both KATmt and KATms (Figs. 1c and S1b and c, respectively). Lys^{231} residue was mutated to Arg (K231R), to generate an acetylation-defective protein, and to Gln (K231Q), to generate acetylated-lysine mimic and both yielded distinctly low acetylation from KATms (Fig. 1d) or KATmt (Fig. S2a) by Western blot analysis. CD spectroscopy analysis confirmed that both the mutated TcrX proteins retained their secondary structure (Fig. S2b).

TcrX phosphorylation and crosstalk are modulated by acetylation

Given that the role of the RR protein as a transcription factor is altered by phosphorylation, from an activated SK protein, we tested the impact of acetylation on phosphorylation reaction using wild-type, acetylated or mutated proteins. Both cognate and non-cognate phosphotransfer reactions were probed, which occurs through SK TcrY or SK MtrB, respectively. The phosphorylation of TcrX through GST-tagged TcrY, its cognate SK, was examined in a time course experiment as reported previously [42]. Autophosphorylated TcrY (time course shown in Fig. S3a) rapidly transferred ^{32}P-labeled phosphoryl moiety to TcrX, evident by the decay of phosphoryl signal from TcrY and appearance on TcrX (Fig. 2a). Similarly, the presence of cross-phosphotransfer from non-cognate SK MtrB to TcrX was also observed within 5 min (Fig. 2b) (MtrB autophosphorylation shown in Fig. S3b) as reported previously [8].

Next, we examined the effect of TcrX acetylation on the phosphotransfer reactions. Toward this, TcrX was acetylated using KATms and the phosphotransfer from TcrY was evaluated in a time course experiment. The outcome revealed many interesting things; first, the rate of decay of TcrY ~ P was lesser in the presence of acetylated TcrX, and second, TcrX ~ P was more stable when acetylated (Fig. 2c, compare right with left panels). This indicated that acetylation modulates the rates of two processes, *viz.* phosphotransfer from SK to RR and dephosphorylation of TcrX. Contrary to this, when the non-cognate SK MtrB was used as a donor in the phosphotransfer time course assay, reduced phosphotransfer to the acetylated TcrX protein compared to the wild-type protein was recorded (Fig. 2d, panel i *versus* ii). Densitometric quantitation confirmed that acetylation enhances TcrX phosphorylation from TcrY during the 360-min duration and reaches 3-fold higher levels (Fig. 2e), while phosphorylation from MtrB is reduced by almost 90% upon acetylation at all time points (Fig. 2f). Rapid decay of phosphoryl moiety from TcrX in non-acetylated state suggested that it could be acting as a signaling sink, which is plugged when it is acetylated.

To further analyze the effects recorded with MtrB, a time course experiment was done with acetylation-defective TcrX K231R and acetylation-mimic TcrX K231Q mutant proteins. Here also, reduction in phosphotransfer for the acetylation-mimic TcrX K231Q protein was recorded (Fig. 2d, panel iv) unlike for acetylation-defective TcrX K231R (Fig. 2d, panel iii). However, inhibition was also observed in a control reaction containing KATms, but lacking acetyl-CoA (Fig. S3c, panels i–iii), suggesting possible stearic hindrance between acetyltransferase and the SK, which interferes with the phosphotransfer reaction. However, in experiments with the mutant proteins, where no acetyltransferase was used, an effect of acetylation on suppression of phosphotransfer was clearly established. Here, significantly lower phosphorylation for TcrX K231Q was recorded at the 30-min time point compared to wild-type TcrX or TcrX K231R (acetylation-defective) proteins (Fig. S3c, panel iv *versus* panel v).

To clearly evaluate the effect of acetylation, we prepared tagless acetylated TcrX (separated from acetyltransferase, described in detail in the Supplementary Methods section), and used it in the phosphotransfer reaction in the presence or absence of KATms. We recorded distinctly enhanced phosphorylation of TcrX as well as TcrY when TcrX was acetylated (Fig. S4a, panel i *versus* panel ii). Measurement of decay kinetics of TcrY confirmed that acetylated-TcrX ~ P is more stable than non-acetylated TcrX ~ P, thereby proposing a phosphatase activity suppressing role for acetylation (Fig. S4a). Further phosphotransfer reactions with non-cognate SK MtrB confirmed that the presence of acetylation alone limits the amount of phosphorylated TcrX (Fig. 3a, panel i *versus* ii). For all reactions, it was confirmed that tagless TcrX was stably acetylated even after separation from the acetyltransferase (Fig. S4a, bottom panel). An interesting observation was that the effect of acetylation on TcrX on phosphotransfer reaction was partially mimicked in the reactions containing only the acetyltransferase

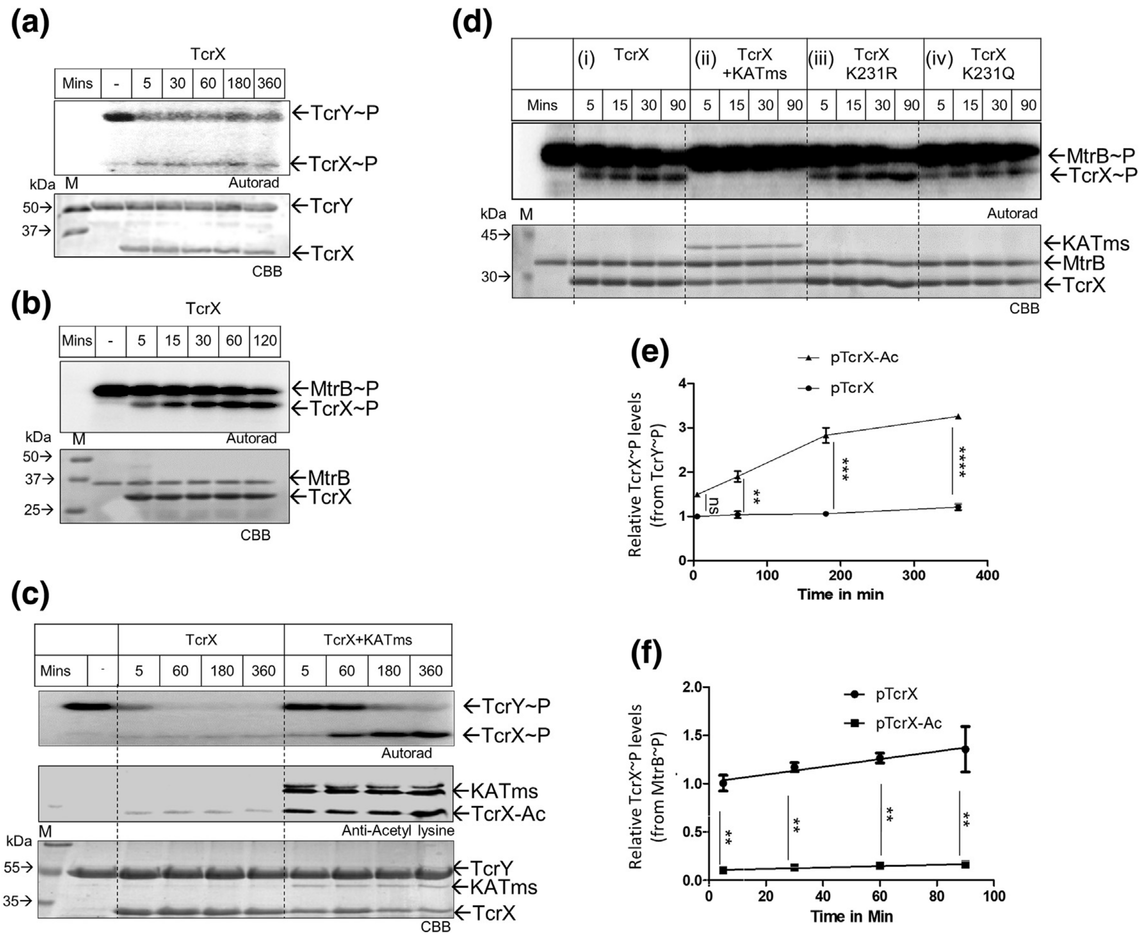
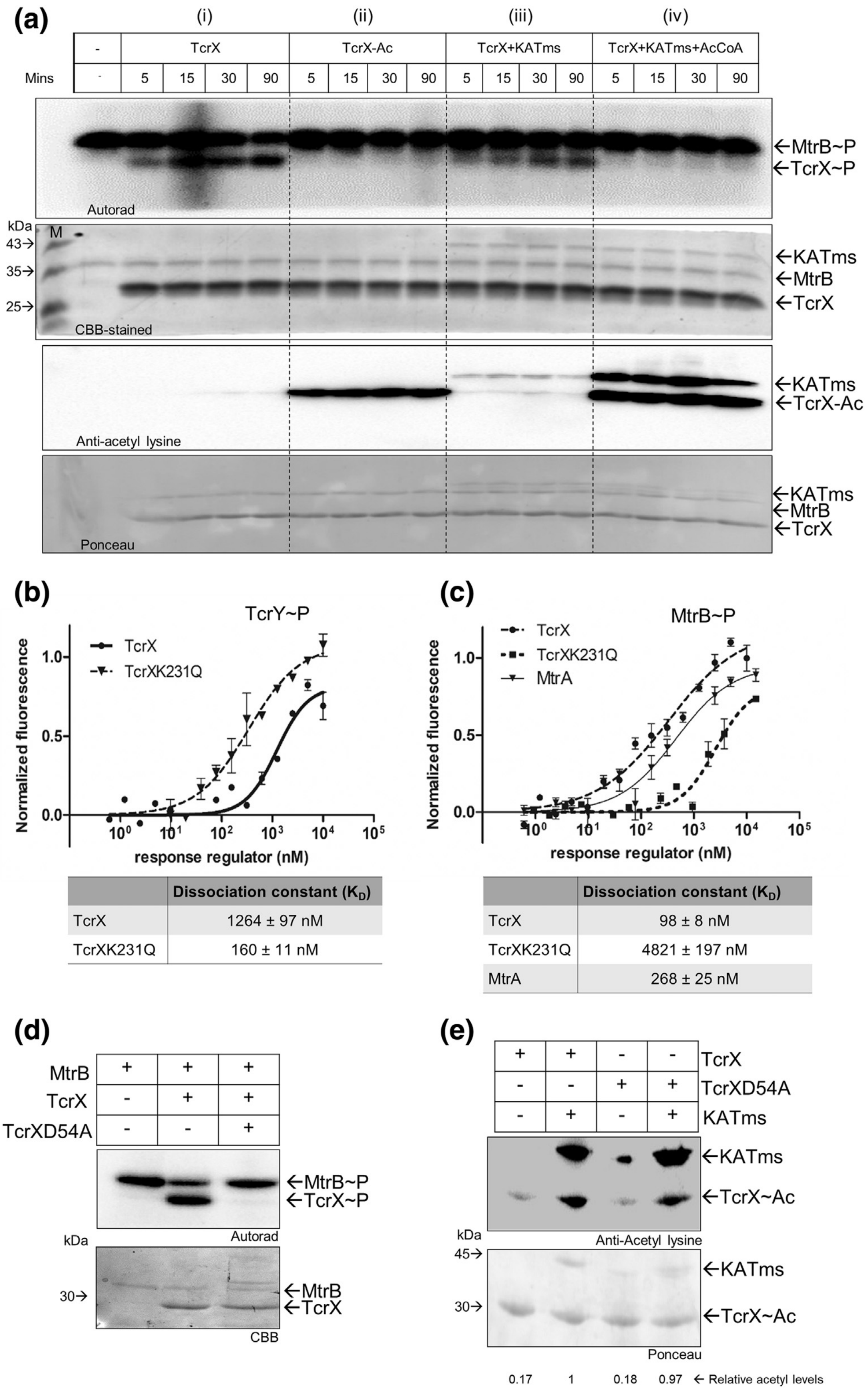


Fig. 2. TcrX phosphorylation analysis. (a) Time course analysis of phosphorylation of TcrX through phosphotransfer from cognate SK TcrY. (b) Time course analysis of phosphorylation of TcrX through non-cognate SK MtrB. For both panels a and b, top, autoradiogram and bottom, CBB-stained gel. (c) Phosphotransfer time course analysis to study the effect of acetylation on TcrX phosphorylation from SK TcrY. The assay was performed using wild-type or acetylated TcrX. Top, autoradiogram; middle, anti-acetyl blot and bottom, CBB stained gel (M, marker). (d) Phosphotransfer time course analysis to study the effect of acetylation on TcrX phosphorylation from non-cognate SK MtrB. The assay was performed using (i) wild-type TcrX, (ii) acetylated TcrX, (iii) acetylation-defective TcrX K231R and (iv) acetylation-mimic TcrX K231Q. Top, autoradiogram and bottom, CBB stained gel (M, marker). (e) Quantitative measurement of TcrX phosphorylation at various time points through cognate SK. (f) Quantitative measurement of TcrX phosphorylation at various time points through non-cognate SK MtrB. For panels e and f, amount of signal recorded (densitometrically determined) for non-acetylated TcrX at 5-min time point was taken as 1, and the rest were normalized with respect to that. The p values calculated using paired Student's t test with respect to non-acetylated TcrX at same time point ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$). For all experiments (n = 3).

enzyme for both TcrY (Fig. S3a, panel iv) and MtrB (Fig. 3a, iii panel), independent of the acetylation status of the RR proteins. The exact mechanism of this effect is, however, not clear, and further studies would be needed to understand the interplay of RR proteins with acetyl transferase enzyme.

To probe whether the key observations made by analyzing phosphorylation status of acetylated TcrX from cognate TcrY and non-cognate MtrB are reflected on the binding affinities of the interacting SK–RR pairs, we analyzed the interaction between various proteins using microscale thermophoresis (MST) [43]. For this, we generated GFP-tagged TcrY and MtrB proteins, which were confirmed to be biochemically active in autophosphorylation and phosphotransfer assays (Fig. S5a and b, respectively) and then analyzed their interaction with wild-type or acetylation-mimic TcrX protein. For SK MtrB, interaction with its cognate RR MtrA was also analyzed in parallel. Interaction studies revealed that the equilibrium dissociation constant for phosphorylated TcrY and non-acetylated TcrX protein was 1264 ± 97 nM (Fig. 3b), 10 times higher than the acetylation-mimic TcrX K231Q protein (K_D = 160 ± 11 nM) (Fig. 3b), demonstrating that the TcrY interacts strongly with the acetylated TcrX than with the non-acetylated protein.



	Dissociation constant (K_D)
TcrX	1264 ± 97 nM
TcrXK231Q	160 ± 11 nM

	Dissociation constant (K_D)
TcrX	98 ± 8 nM
TcrXK231Q	4821 ± 197 nM
MtrA	268 ± 25 nM

Fig. 3 (*legend on next page*)

Analyses with phosphorylated MtrB revealed that the dissociation constant for TcrX was 98 ± 8 nM (Fig. 3c), 40 times lower than acetylation-mimic TcrX K231Q protein (Fig. 3c) (K_D = 4821 ± 197 nM), indicative of higher affinity for non-acetylated TcrX. Interestingly, the relative dissociation constant of the cognate interaction between phosphorylated MtrB and MtrA was 268 ± 25 nM (Fig. 3c), between the rates of MtrB interaction with various TcrX proteins. Collectively, these binding studies prove that the preference of the RR TcrX toward the noncognate SK MtrB is higher compared to cognate SK TcrY under deacetylated condition and is reversed when acetylated, supporting the phosphotransfer modulation observed through time course experiments. These observations strengthen the finding that TcrX acts as an acetylation tunable sink for MtrB phosphate.

Given that acetylation modulates changes in the phosphorylation and interaction of the RRs with SKs, we next probed if changes in phosphorylation proficiency can alter their acetylation. For this, phosphorylation-defective TcrX protein was generated by mutating the conserved aspartate Asp^{54} [42] to alanine (Fig. 3d). Analysis of acetylation potential of the phosphorylation-defective TcrX-D54A protein through KATms revealed no change in its acetylation status (Fig. 3e). We also examined if acetylation alters the phosphorylation potential of MtrA from non-cognate SK TcrY in phosphotransfer assays. However, acetylated MtrA did not accept the phosphate group from TcrY, thereby retaining its fidelity (Supplementary Fig. S5c).

Acetylation of RR MtrA also modulates its phosphorylation

Given that acetylation of TcrX modulates its phosphorylation levels from cognate SK (above), we next tested if a similar effect is recorded for the RR MtrA. First, the acetylation of MtrA through KATms was validated (Fig. 4a), followed by MS/MS analysis to identify Lys^{110} as the acetylation site (Figs. 4b and S1d and e). Lys^{110} is present in the N-terminal domain of the MtrA protein and can affect its phosphorylation based on the crystal structure reported previously [44]. Next, the effect of acetylation on phosphorylation of MtrA through its cognate SK MtrB was evaluated in a phosphotransfer time course experiment and distinct enhancement in phosphorylation was recorded (Fig. 4d and e) similar to TcrY to TcrX phosphotransfer reaction. Given that we had observed that KATms interferes in this reaction, here also we generated tagless MtrA similar to TcrX (as described above), and a phosphotransfer time course was performed from MtrB to MtrA. Acetylated MtrA showed enhanced phosphorylation from MtrB at all time points (Fig. S6a), thereby demonstrating that acetylation enhances phosphotransfer. The addition of KATms mimicked the effects recorded with acetylated MtrA (Fig. S6a, panels iii and iv). These findings demonstrated that acetylation augments the specificity of the phosphotransfer reaction between cognate SK and RR pair and shields it against crosstalk.

Another observation in the MtrA reaction was the presence of stable phosphorylated MtrA when it was acetylated similar to that recorded for TcrX. To understand the basis of this stability, radioactive TLC analysis was performed to record the levels of the Pi released. As evident from Fig. S6b, in the presence of acetylated MtrA, higher residual ATP was present, and less Pi was obtained, indicative of reduced dephosphorylation of acetylated MtrA, consequently less utilization of ATP by MtrB (Fig. S6c). This experiment suggested a role of acetylation of RRs in modulating the rates of both the phosphotransfer and phosphatase reactions in the TCS signaling, a unique finding where both phosphorylation and dephosphorylation of RR are modulated by acetylation.

Acetylation of TcrX inhibits its DNA-binding activity

Given that acetylation altered the phosphorylation of TcrX protein, we evaluated if the DNA-binding function of the RR TcrX is also affected. For this, the effect on a 508-bp autoregulatory promoter region of *tcrXY* [45] was tested in an electrophoretic mobility shift assays (EMSAs). EMSA performed in the presence of increasing amounts of TcrX protein showed distinct mobility shift (Fig. 5a) as reported

Fig. 3. Analysis of the effect of acetylation on TcrX phosphorylation and interaction. (a) Time course analysis of phosphotransfer from SK MtrB to tagless TcrX. Panel (i) TcrX, (ii), acetylated TcrX, (iii) TcrX with KATms and (iv) TcrX with KATms and acetyl CoA as described in Fig. 2d. Top image, autoradiogram; second image, CBB stained gel; third image, anti-acetyl blot; and bottom image, Ponceau stained blot (M, marker; n = 3). (b) MST measurements for determination of interaction affinities of phosphorylated TcrY-GFP with TcrX and TcrX K231Q. (c) MST measurements for determination of interaction affinities of phosphorylated MtrB-GFP with TcrX, TcrX K231Q and cognate RR MtrA. All graphs were best fit to mean ± SEM from three independent experiments. The K_D values determined by MST as indicated in the table below panels b and c. (d) Phosphorylation analysis of wild-type and D54A mutant of TcrX protein using MtrB as a phosphodonor species. The reaction was performed as described in Fig. 2d. (e) Analysis of acetylation status on TcrX D54A mutant protein. Analysis was done as reported in Fig. 1b. Top, image of blot probed for acetylated lysine, and bottom, Ponceau stained blot. Numbers indicate quantitative measurement of acetylation on the respective TcrX protein. Amount of signal recorded for acetylated TcrX was taken as 1, and the rest were normalized with respect to that (n = 3).

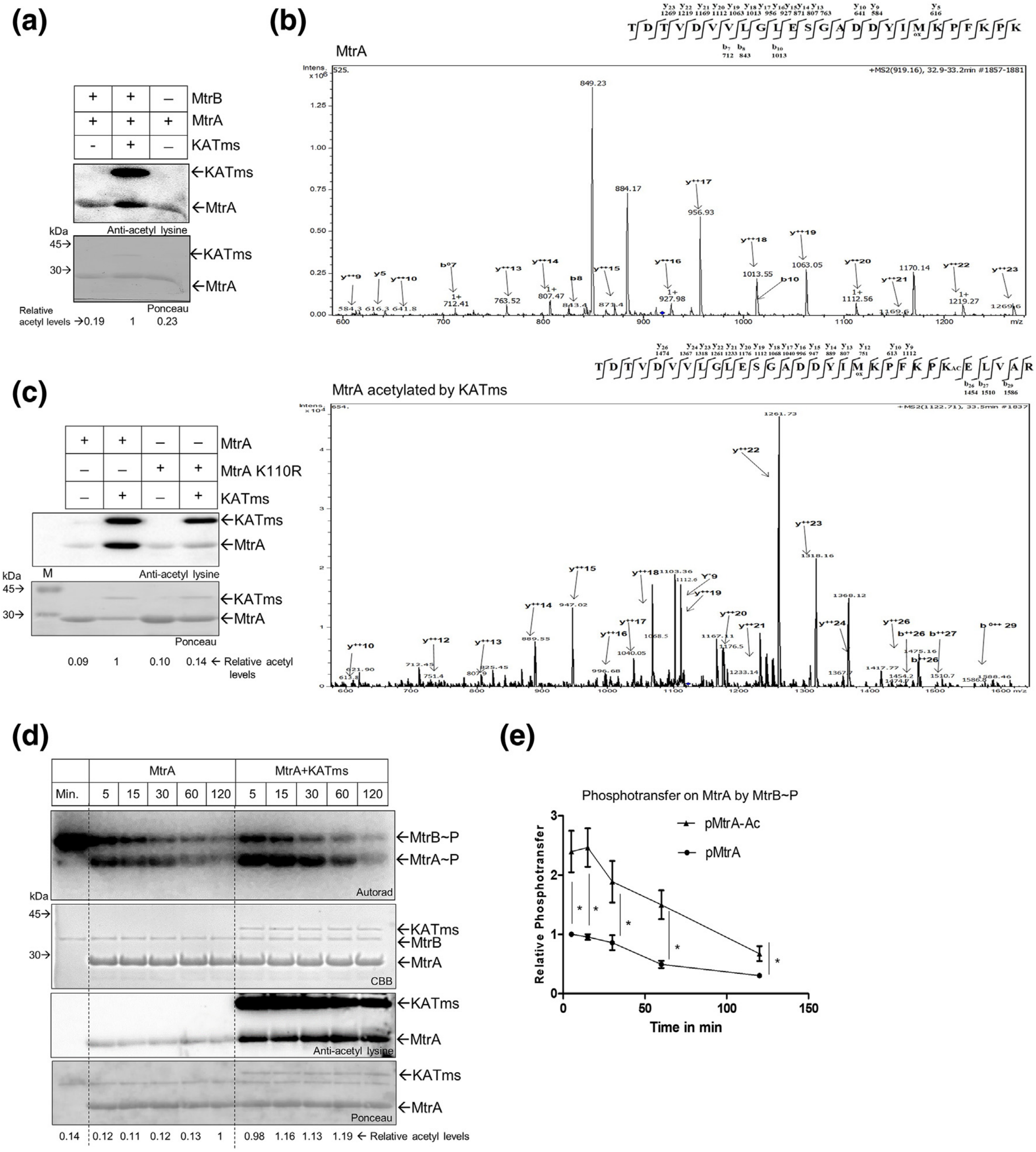


Fig. 4. Identification and characterization of acetylation of RR MtrA. (a) Acetylation of MtrA using KATms under *in vitro* conditions, identified by Western blotting for acetylated lysine. Top, blot probed with anti-acetyl lysine antibody, and bottom, Ponceau stained blot. Numbers indicate quantitative measurement of acetylation on the respective proteins, normalized to acetylated MtrA as 1 (n = 3). (b) MS/MS analysis for identification of acetylation site on MtrA by KATms (lower panel). (c) Mutagenesis to confirm Lys^{110} as the acetylation site in MtrA. The wild-type and mutant K110R MtrA proteins were probed for acetylation using KATms as described in Fig. 1d. Top, blot probed with anti-acetyl lysine antibody, and bottom, Ponceau stained blot. Numbers indicate quantitative measurement of acetylation on the respective proteins, normalized to acetylated MtrA as 1 (n = 3). (d) Time course analysis phosphotransfer from MtrB to MtrA and analysis of the effects of acetylation. The assay was performed as described in Fig. 2a. First panel, autoradiogram; second panel, CBB stained gel; third, image of blot probed for acetylated lysine; and fourth, Ponceau stained blot. Numbers indicate quantitative measurement of acetylation on the respective proteins, normalized to acetylated MtrA at 5 min as 1 (n = 3). (e) Quantitative measurement of phosphorylated MtrA protein at various time points with respect to its acetylation status through cognate SK MtrB (data from experiments reported in panel d). Signal recorded for unacetylated MtrA at 5-min time point was taken as 1, and the rest were normalized with respect to that. *p* Values calculated using paired Student's *t* test with respect to non-acetylated MtrA at same time point ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$). For all experiments, n = 3 independent biological replicates.

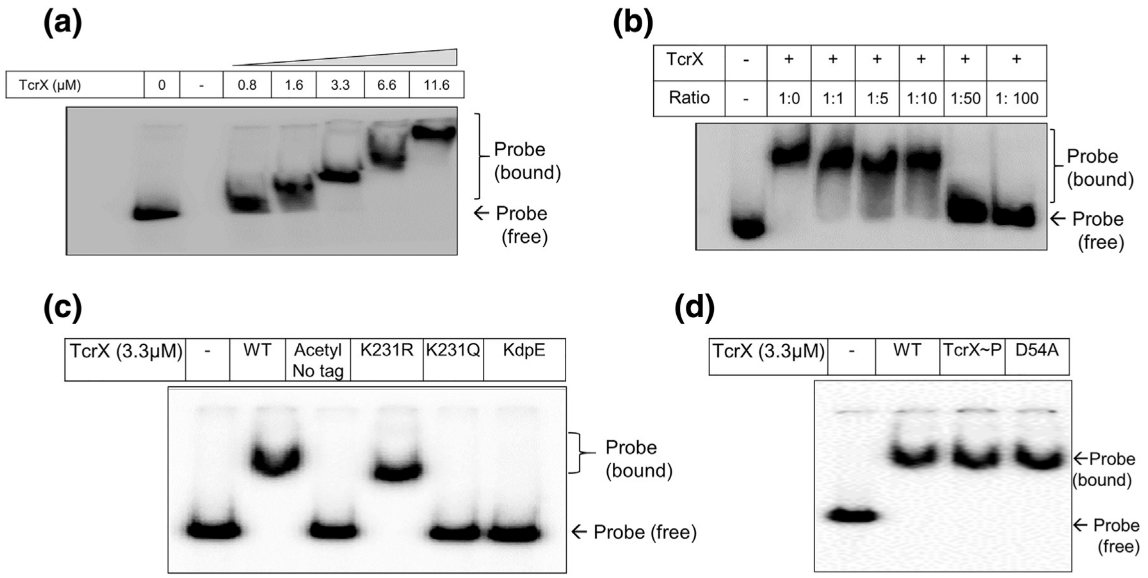


Fig. 5. Effect of acetylation on DNA-binding activity of TcrX. (a) DNA-binding activity of TcrX was assessed by EMSA using 508 bp of labeled *tcrX* promoter region as described in the Materials and Methods section. Increasing amounts of TcrX protein (as indicated) were used. (b) Cold competition assay for confirming the specificity of binding. ^{32}P-labeled and -unlabeled DNA was mixed in ratios as indicated, and EMSA was performed using TcrX protein. (c) Effect of acetylation on DNA-binding ability of TcrX. Wild-type, mutant or acetylated TcrX protein (as indicated) was used in the EMSA. KdpE RR was used as a negative control in the EMSA reactions. (d) Effect of phosphorylation on DNA-binding ability of TcrX. Wild-type phosphorylated (TcrX~P) or D54A mutant (as indicated) was used in the EMSA. For all experiments, the position of bound and unbound DNA probe is indicated. n = 3 biological replicates.

previously [45], which was validated by cold competition assay (Fig. 5b). To test the effect of acetylation on DNA-binding activity of TcrX, it was acetylated using KATms, separated from KATms as reported above and tested in EMSA. This had to be done as KATms also interfered with the DNA-binding ability of TcrX (Fig. S7a). Surprisingly, the acetylated protein failed to bind DNA (Fig. 5c, lane 3), similar to the acetylation-mimic TcrX K231Q mutant, but unlike the acetylation-defective TcrX K231R protein (Fig. 5c, lane 5). As a control, binding of another RR KdpE to the TcrX promoter region was tested and was found to be absent (Fig. 5c, last lane). We also examined if there is a difference in the affinity of acetylated TcrX for DNA binding by evaluating increasing amounts of proteins in EMSA, and significantly reduced shift was observed compared to non-acetylated TcrX (Fig. S7b). When the effect of phosphorylation of TcrX on DNA binding was analyzed using either phosphorylated TcrX, generated through TcrY~P (after removal of TcrY) or using phosphorylation defective TcrX D54A, no change in the DNA-binding ability was observed and the TcrX protein bound to the DNA similar to its non-acetylated state (Fig. 5d) [45]. Here also change in affinities was tested by analyzing various amounts of TcrX~P in EMSA, and no change in the DNA-binding in response to TcrX phosphorylation was observed (Fig. S7c) as reported previously [45]. These findings propose yet another role for acetylation in the regulation of TcrX function, which is independent of its phosphorylation status.

A similar analysis was done using RR MtrA, and the effect of acetylation and phosphorylation on its DNA-binding ability was tested on the promoter region of a known target, *dnaA* [46]. Here, we observed that while phosphorylation of MtrA enhanced its binding to the promoter region, as reported before [46], no effect of acetylation on DNA binding was observed (Fig. S8a) and no affinity changes were also recorded by concentration titration also (Fig. S8b). In addition, binding of MtrA on the *tcrXY* promoter region was tested and was found to be absent (Fig. S8c) negating any cross-regulation.

In vivo analysis of TcrX acetylation and analysis of its effect on gene expression and crosstalk in Mycobacteria

To validate our observations *in vivo*, we first probed for the presence of TcrX acetylation *in vivo*, for which the wild-type TcrX protein from *M. tuberculosis* was overexpressed in either wild-type *M. smegmatis* mc^{2}155 or in acetyltransferase mutant strain (ΔKATms) [47] using pMV261 expression vector, which drives expression through a strong *hsp60* gene promoter. Expression of TcrX protein in the wild-type strain was observed in two different growth conditions using anti-TcrX antibody. Interestingly, the antibody did not detect any endogenous expression of TcrX protein in the wild-type strain, indicating that the protein is either not expressed or expressed at very low conditions levels in the basal conditions (Fig. 6a, lanes 2 and 4). Because of the autoregulatory role of TcrX, we also observed an increase in the levels of endogenous TcrX protein of *M. smegmatis* mc^{2}155 (ortholog, TcrXms, 27.2 kDa) in the presence of the introduced TcrXmt (25.8 kDa) (Fig. 6a, lanes 3 and 5). When the acetylation of the overexpressed protein was analyzed in wild-type or the KATms defective strain using specific anti-acetyl lysine antibody, as anticipated the TcrXmt was found to be acetylated in wild-type *M. smegmatis*, but not in ΔKATms strain (Fig. 6b), confirming that TcrX is indeed a substrate for KATms. However, no acetylation of the endogenous TcrXms protein was detected.

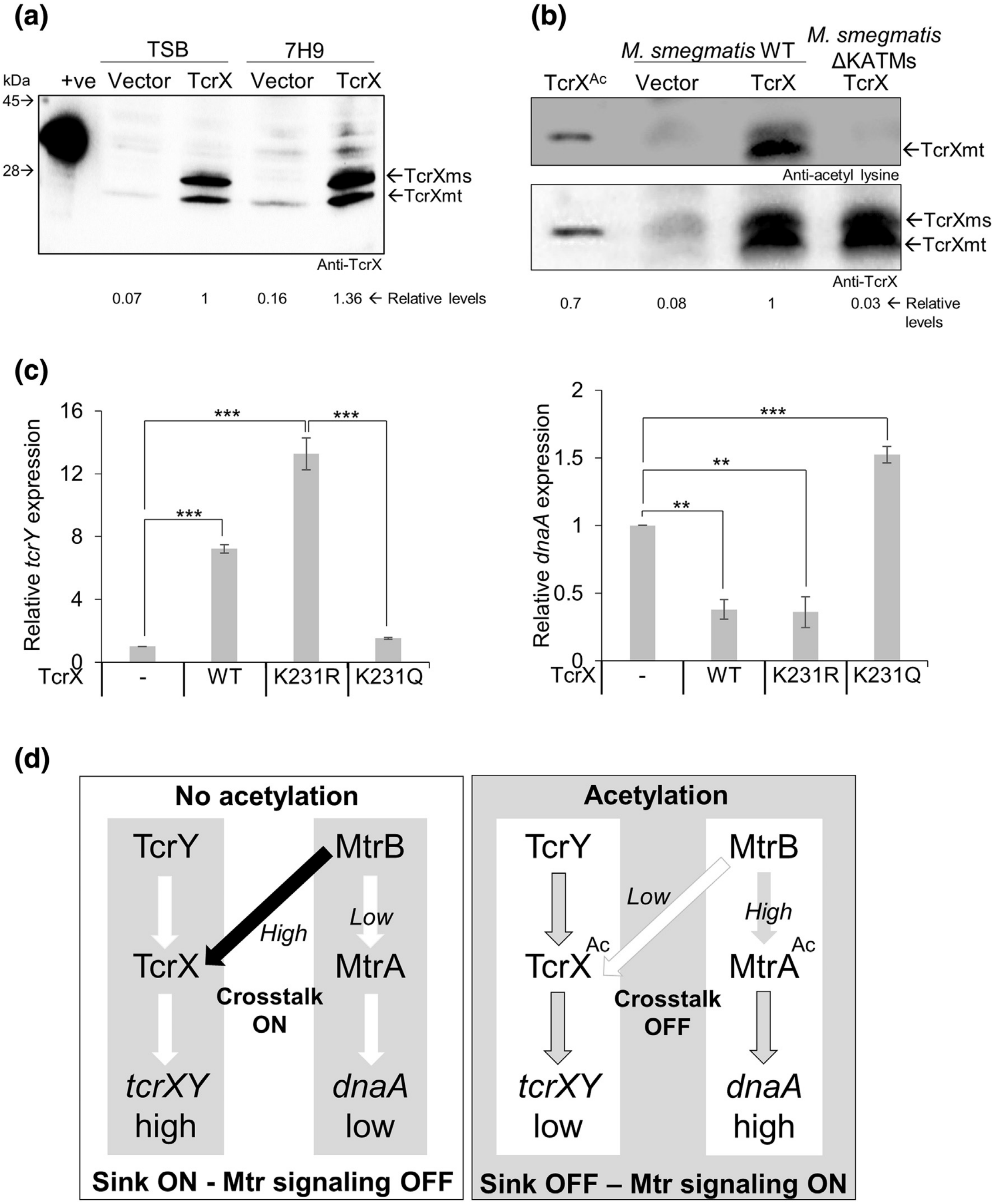


Fig. 6 (*legend on next page*)

When the protein sequence of TcrXms was aligned with TcrXmt, we observed that the Lys^{231}, which is the site of acetylation in TcrXmt, is replaced with arginine in TcrXms (Fig. S9a), accounting for lack of acetylation. In addition, the absence of acetylation in overexpressed TcrX K231R mutant protein confirmed that it is the site of acetylation *in vivo* as well as in the *in vitro* conditions (Fig. S9b).

To study the effect of RR acetylation in *in vivo* conditions, wild-type, acetylation-defective (K231R) or acetylation-mimic (K231Q) TcrX proteins were overexpressed in *M. tuberculosis* H37Ra strain. Given that acetylation of TcrX affects its phosphotransfer ability from cognate and non-cognate SK and also its autoregulation, we probed for changes in expression of downstream target genes of TcrXY and MtrAB. Expression levels of the *tcrXY* operon, autoregulatory target of TcrX [45], were analyzed using *tcrY* gene, and its expression increased when wild-type TcrX or acetylation-defective TcrX was overexpressed (K231R), but not by acetylation-mimic mutant TcrX K213Q (Fig. 6c, left panel), thereby validating our *in vitro* observations of negative autoregulation by TcrX acetylation.

Next, to test if the increase in the TcrX levels and its acetylation allow us to record crosstalk with MtrB, the expression of genes regulated by the MtrAB TCS was evaluated. We aimed to see if RR TcrX can act as a sink for phosphorylated MtrB and if overexpression of wild-type TcrX or acetylation-defective TcrX K231R results in sequestration of the MtrB phosphoryl signal, thereby limiting the phosphorylation of its cognate RR MtrA. Consequently, reduced MtrA ~ P levels will result in lowering of the levels of target genes such as *dnaA*, which are positively regulated by MtrA ~ P [48]. In accordance with our hypothesis, reduction in the levels of *dnaA* was recorded in the presence of acetylation-defective TcrX K231R, but not when acetylation-mimic TcrX K231Q was overexpressed (Fig. 6c, right panel), reaffirming that acetylation enhances signal fidelity and reduces crosstalk. The requirement of cross-phosphorylation in this crosstalk was also examined by overexpressing TcrX protein mutated in both phosphorylation (Asp^{54}) and acetylation (Lys^{231}) sites. Overexpression of this protein did not alter the expression of *dnaA*, and levels similar to parental wild-type strain were recorded (Fig. S9c), confirming that the changes in the expression are mediated by cross-phosphorylation or crosstalk. We ruled out the presence of cross-regulation or shared regulons [3,49] by analyzing direct binding of TcrX on *dnaA* promoter and MtrA to the *tcrXY* promoter by EMSA. While MtrA ~ P bound to *dnaA* promoter and a distinct shift in mobility was observed in EMSA, no binding of TcrX to *dnaA* was recorded (Fig. S8a, lane 8). Likewise, binding of MtrA protein to the *tcrX* promoter was also found to be absent (Fig. S8c).

Overall, we show that phosphotransfer dynamics and associated crosstalk in TCSs are tuned by acetylation of participating RR proteins, which modulates their signaling fidelity. Our findings thus provide the first report of metabolic regulation of signaling specificity (by modulating levels of phosphorylated RRs and phosphotransfer rates) and persistence (by modulating phosphatase activity) in two-component signaling systems. We also show that while phosphorylation is one of the most popular mechanisms of regulating RR activity, acetylation can also regulate their activities, both at the level of DNA binding and phosphorylation.

Discussion

The classical model for two-component signal transduction pathways involves regulation of gene expression changes in response to extracellular cue/s through phosphorylation-mediated switch in the DNA-binding ability of the RR protein. This phosphorylation-mediated switching of RR activity is widely observed and is the accepted standard for regulation of TCS activity. However, for some RRs, it has been observed that while their deletion affects cellular physiology, defects in their phosphorylation site do not [48]. This suggests a phosphorylation independent mechanism for regulation of RR activity which modulates their roles as transcription factors.

Fig. 6. Identification of *in vivo* acetylation on TcrX and physiological effect analysis. (a) TcrX expression was analyzed in *M. smegmatis* mc^2155 strain carrying either vector pMV261 alone or expressing wild-type TcrX protein grown in either tryptic soya broth (TSB) or Middlebrook 7H9 medium (7H9) by Western blotting (TcrXmt, *M. tuberculosis* TcrX; TcrXms, *M. smegmatis* TcrX; +ve control, purified TcrX protein). Numbers indicate quantitative measurement of acetylation on the respective proteins, normalized to TcrXmt overexpressed in TSB media as 1 (n = 3). (b) Acetylation status of overexpressed TcrX protein. *M. smegmatis* strains (as mentioned) containing various plasmids for acetylated lysine and TcrX protein. Top, blot probed for acetylated lysine; bottom, for TcrX protein. Numbers indicate quantitative measurement of acetylation on the respective proteins, normalized to acetylated TcrXmt as 1 (n = 3). (c) Analysis of gene expression in *M. tuberculosis* H37Ra strains expressing various TcrX proteins (as mentioned). Left, expression changes in *tcrY* gene, a direct target of TcrX RR, and right, expression changes in the *dnaA* gene, a direct target of RR MtrA. The gene expression was normalized to the expression levels of 16S rRNA, followed by the expression levels in the strain carrying only the vector. For all experiments, n = 3 biologically independent experiments. (d) Model of regulation of crosstalk between TcrXY and MtrAB TCSs by acetylation. The terms low and high indicate the quantum of signal propagation through the cascade, which is reflected on the expression of downstream genes as shown in Fig. 6c.

Acetylation as a post-translational modification is known to affect the activities of various RRs. In *Salmonella typhi*, acetylation of RR PhoP and RcsB in *E. coli* reduces their affinity of binding to target DNA [19,25]. However, the effect of acetylation on the phosphorylation has been reported only for CheY [24,50]. Given that acetylation as a post-translational modification is coupled to the energy homeostasis of the cell, it is reasonable to expect that acetylation can link gene expression changes to the carbon metabolism in the cell. This in the context of the pathogenic lifestyle of *M. tuberculosis* has implications in metabolic adaptations influencing its virulence and survival.

The studies presented here show that the acetylation affects the activities of RR proteins, which act as transcription modulator, by not only affecting their DNA-binding activity but also by modulating their phospho-acceptance potential. This observation *per se* is not very surprising, but the novel finding is that acetylation status of the RR TcrX modulates the crosstalk and fidelity between TCS proteins. It has been recently reported that the TCS proteins of *M. tuberculosis* participate in high crosstalk [8]. In the phosphotransfer landscape identified in *M. tuberculosis*, TcrX protein is a promiscuous RR capable of accepting phosphate from more than one SK; that is, it can serve as a signal integration node. Similarly, the SK MtrB SK is capable of transferring phosphoryl moiety to multiple RRs, including TcrX. The existence of this crosstalk proposed the presence of novel signaling landscapes, wherein kinetic preferences of phosphorylated SKs to various RRs should dictate the final adaptive response. While it is anticipated that the substrate preference is governed by the inherent inter-protein interface compatibility [51] or by absolute numbers of participating molecules available in the reactions, it would also depend on other factors that can alter the interaction between the SK and RR proteins, which includes the presence of other post-translational modifications, which can alter the interface affinity and the prevailing phosphorylation status of the proteins.

It was observed here that the ability of TcrX to accept and retain phosphate from its cognate SK improved considerably when it was acetylated. While very rapid decay of phosphoryl signal was recorded in its cognate SK TcrY and for non-acetylated RR TcrX, similar to what has been seen for many RRs [8], signal sustenance was only seen when TcrX was acetylated indicative of modulation of phosphatase activity as well. This was opposite to what was observed when the non-cognate SK MtrB was used as a donor and the phosphotransfer on acetylated TcrX was lower compared to the non-acetylated form (Fig. 3A). Similarly, for RR MtrA, its acetylation also enhanced its phosphorylation from its cognate SK MtrB (Fig. 4d). This modulation in the direction of phosphate flow by acetylation (stronger for cognate SK–RR when RR is acetylated) allows TcrX to serve as an acetylation sensitive signaling switch for MtrB ~ P, allowing control of the signaling outcomes based on the nutrition status of a cell. In the case of TcrY➔TcrX transfer, the insulation is further strengthened in the acetylated state by increased stability of phosphorylated TcrX and TcrY, which limits the amount of free TcrX available for accepting signaling from MtrB. In addition, further reduction in the levels of TcrXY takes place as acetylated TcrX is a negative regulator of its own levels, which enhances specific signaling and further limits crosstalk.

In a situation where acetylation is low, TcrX acts as a sink for MtrB phosphate and suppresses the MtrA regulon. The absence of acetylation not only reduces MtrB➔MtrA signaling, but it also invokes various other mechanisms to keep the signal to MtrA low, which includes decreased signaling transmission from TcrY➔TcrX, and rapid dephosphorylation of TcrX and TcrY, which generates sufficient quantity of free TcrX to act as a sink. Furthermore, free non-acetylated TcrX also increases its own amount by positively regulating its autoregulatory axis, thereby ensuring that MtrB signal is not transmitted to MtrA (Fig. 6d). In agreement with this, it has been shown previously that MtrB deletion strain is viable [40], and sustained phosphorylation of MtrA is deleterious to mycobacterial cells and a mutant MtrA protein (Y102C), which shows sustained phosphorylation, cannot be overexpressed [52,53]. What is also interesting about MtrA acetylation site is that it lies in the N-terminal domain, close to its phosphorylation site. Similarly located site in CheY was reported to inhibit its phosphorylation [24], but for MtrA, it facilitates phosphorylation, probably due to different domain arrangements present in these proteins. Acetylation in the C-terminal domain of many RRs has been observed and has been shown to affect their DNA-binding activities, for example, in RcsB protein in *E. coli* and PhoP protein in *Salmonella*. For CheY protein of *E. coli*, multisite acetylation affects its protein–protein interaction ability [19,25,54].

We thus provide the first known example of modulation of phosphotransfer fidelity from SK to RR through a post-translation modification of RR protein. Our findings not only demonstrate that acetylation affects phosphotransfer reaction but also show that acetylation status of RRs has the potential to establish alternate signaling landscapes, wherein the reliability of the phosphotransfer reaction dictates the crosstalk present in the system (Fig. 6d). Findings from our *in vivo* studies, wherein we modulated the specific phosphorelay between MtrB and MtrA and thereby their physiological roles, using various TcrX protein/s as a tunable switch provide strong support to our model.

Materials and Methods

Materials

All the media chemicals, biochemicals and protein reagents were purchased from Sigma Merck (St. Louis, USA), antibiotics and DTT (Dithiothreitol) from Goldbio (USA), protein markers from Thermo Fisher (USA), agarose GSH resin from GE Healthcare (USA), and Ni^{+2}-NTA resin from Roche Diagnostics (USA). Restriction Enzymes were from Thermo Fisher (USA). Primers were synthesized from Bioserve (India), and $\gamma^{32}P$ ATP (>3500 Ci/mmol) was purchased from BRIT-Jonaki (India).

Bacterial strains and recombinant plasmid construction

Cloning/mutagenesis and protein overexpression were carried out in *E. coli* strain DH10β and BL21 Arctic Express™ (Agilent Technologies, USA) or $SP850cyc^{-}$ strain for acetyltransferase expression [47]. The strains were grown in LB medium with 100 µg/ml ampicillin, 50 µg/ml of gentamycin and 10 µg/ml of kanamycin, respectively. The list of primers used for PCR, cloning or mutagenesis is provided in Supplementary Table 1. Recombinant plasmids used for protein overexpression were reported previously [8,47], and details of new constructs are described in Supplementary Methods and Table S1.

Protein expression and purification

For protein overexpression, *E. coli* cells containing the expression plasmids for TcrX, TcrY, MtrB, MtrA, GFP-tagged TcrY or MtrB, MSMEG_5458, Rv0998, MSMEG_5175 (deacetylase) [47] or rTEV were grown at 37 °C in 200 ml of 2× YT or Terrific broth to an OD_{600} > 1.0 followed by induction with IPTG (0.1–1.0 mM). The culture was further grown for 15–20 h at 12–15 °C for protein expression. Cells were harvested by centrifugation and stored at −80 °C until use. For purification of 6× His-tagged and GST-tagged proteins in soluble conditions, the protocol described previously was followed [8,47].

Phosphorylation assays

Phosphorylation assays were essentially performed as per the protocol described previously [8], and details are provided in supplementary methods.

Acetylation status analysis

For *in vitro* acetylation, test proteins (3 µg) were incubated in reaction buffer containing 25 mM Tris–Cl (pH 7.4), 100 mM NaCl, 100 µM cAMP, 10 µM acetyl-CoA (100 µM acetyl-CoA for Rv0998) and 0.2 µg of Rv0998/MSMEG_5458 at 37 °C for 4 h. The reactions were terminated by boiling and analyzed by Western blotting using anti-acetyl lysine antibody (1:7500, No. 9441; Cell Signaling Technology Inc., USA), followed by HRPO-conjugated secondary antibody (1:5000) and developed with ECL+ reagent (PerkinElmer, USA) as per manufacturer's protocol.

To identify TcrX as the *in vivo* substrate of KATms, the *M. smegmatis* $mc^{2}155$ or acetyltransferase mutant strain [47] was electroporated with pMV261 vector or pMV261 containing wild-type *tcrX* gene. Colonies were selected on kanamycin containing 7H11 agar (HiMedia, India) media supplemented with 0.2% glycerol, 0.05% tween 80, 10% ADC (albumin, dextrose, catalase) and 25 µg/ml kanamycin at 37 °C. Liquid cultures were grown in Middlebrooks's 7H9 broth (HiMedia, India) supplemented with 0.2% glycerol, 0.05% tyloxapol, 10% ADC and 25 µg/ml kanamycin with shaking at 180 rpm at 37 °C.

For the preparation of whole cell lysates, cultures grown till mid-log phase were washed three times with 1× PBS to remove traces of albumin and media. Cells were resuspended in 20 mM Tris–Cl (pH ~ 7.5), 100 mM NaCl, 10% glycerol, 1 mM PMSF, 1 mM benzamidine hydrochloride and 1% Nonidet-P40. Cells were lysed by bead beating with 0.1 mm zirconia/silica beads (Thomas Scientific, USA), followed by centrifugation for 30 min at 12,000 rpm at 4 °C. Supernatants were collected and protein was estimated by Bradford's reagent. Acetylation status was analyzed by Western blotting as per protocol described above. Anti-TcrX antibody raised in rabbit was used for TcrX expression confirmation (1:1000 dilution).

Acetylation site identification

MS/MS-based site acetylation site identification was done using standard and previously reported protocols [28]. Details are provided in Supplementary methods.

Purification of acetylated RRs. Details of methodology and flowchart of steps utilized to prepare tagless acetylated RRs, free from the KATms and rTEV protease, are described in the Supplementary Methods section.

Phosphorylation and phosphotransfer assays

These reactions were performed as per the protocol described previously [55], and the details are provided in the Supplementary Methods section.

Determination of affinities of phosphorylated SK and RRs using MST analysis

Purified recombinant MtrB-GFP protein (50 nM) was phosphorylated for 1 h at 30 °C using 50 µM

ATP and then mixed with increasing concentrations of titrant cognate RR MtrA (0.61 nM to 15 μM) or non-cognate RR TcrX (0.61 nM to 15 μM) or its mutant TcrX K231Q (0.92 nM to 15 μM). Similarly, TcrY-GFP (50 nM) protein was autophosphorylated and mixed with titrant cognate RR TcrX (0.61 nM to 15 μM) or its mutant TcrX K231Q (0.92 nM to 15 μM). The sample was then loaded into the standard treated capillaries and analyzed using a Monolith NT.115 system (NanoTemper Technologies, GmbH). The blue laser was used for a duration of 35 s for excitation (MST power = 60%, LED power 40%). The data were analyzed using MO Control software (NanoTemper Technologies, GmbH) to determine the dissociation constants (K_D) for interacting proteins.

Electrophoretic mobility shift assay

A 508-bp upstream region of the *tcrYX* operon was PCR-amplified from *M. tuberculosis* H37Rv genomic DNA template using specific primers as reported previously [42] (Supplementary Table 1), and EMSA was performed after labeling the same as per the protocol previously described [56]. Details are provided in Supplementary Methods.

RNA extraction and quantitative gene expression analysis

Exponentially grown cultures of *M. tuberculosis* H37Ra containing either pMV261 vector alone or containing wild-type or mutant TcrX proteins were used for analysis of gene expression changes by RT-PCR. Details of the protocol utilized for RNA extraction, RT-PCR are provided in Supplementary Methods. Expression analyses were performed using three independent biological replicates.

Statistical Analyses

Statistical analyses for significance were performed using Student's *t* test and Mann–Whitney *U* test between control and experimental set only. For all experiments, the number of independent biological replicates used is indicated by “n.”

CRediT authorship contribution statement

Krishna Kumar Singh: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Neerupma Bhardwaj:** Data curation, Methodology, Resources. **Gaurav D. Sankhe:** Investigation, Methodology. **Niveda Udaykumar:** Investigation, Methodology. **Rambir Singh:** Conceptualization, Project administration, Supervision. **Vandana Malhotra:** Conceptualization, Project administration, Supervision. **Deepak Kumar Saini:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Supervision, Writing - original draft, Writing - review & editing.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

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Abbreviations used:
TCS, two-component signal transduction; SK, sensor kinase; RR, response regulator; EMSA, electrophoretic mobility shift assay; MST, microscale thermophoresis; “”, complementarity determining region; NOE, nuclear Overhauser effect; IDR, intrinsically disordered region;

MoRF, molecular recognition feature.

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