The functional diversity and efficacy of proteins inside a cell is enhanced by various post-translational modifications that occur on them. Both prokaryotic and eukaryotic proteins possess more than 300 PTMs that are known to play an important role in regulation, cell signalling, protein degradation and protein-protein interactions. Nα-acetylation is one of the most abundant modifications occurring on approximately 80% of the proteins in eukaryotes. Nα-acetylation play diverse roles inside a cell ranging from protein-protein interactions, protein targeting to organelles, determining half–life of proteins etc. However, Nα-acetylation and its physiological importance in prokaryotes is largely an unexplored phenomenon.

Enzymes responsible for catalysing transfer of acetyl moiety to the N-terminus of the protein are known as Nα-acetyltransferases (NATs). Based on their subunit requirements and substrate specificities NATs have been divided into six families, NatA- NatF, in eukaryotes. However, in bacteria only a couple of NATs are characterized. The bacterial NATs are commonly named as RIM enzymes. These enzymes acetylate ribosomal proteins in a substrate specific manner. Partial Nα-acetylome of *M. tuberculosis* suggests that 28% of mycobacterial proteome is acetylated with higher abundance of NatA and NatE–like substrates. However enzyme(s) responsible for these modifications are not known. The closest homolog of NatA subunit in bacteria is encoded by ORF *Rv3420c*.

In this study, we report the first NAT encoded by ORF *Rv3420c* from *M. tuberculosis* that catalyses the transfer of acetyl-coA on to the N-terminus of peptides/proteins. The other reported NAT namely, Eis is a lysine acetyltransferase that acetylates DUSP16/MKP-7 in *M. tuberculosis* while its ortholog acetylates antibiotic kanamycin in *M. smegmatis*. In our study, we identify that $RimI^{Mtb}$ is a monomer in solution and while it could acetylate N-terminus of ribosomal protein S18 of *S. typhimurium*, N-terminus of putative ribosomal proteins from Mtb could not be acetylated, efficiently. Therefore, we next explored the substrate specificity of $RimI^{Mtb}$. A novel small scale tryptic peptide library (STPL) assay was developed that helped us elucidate that $RimI^{Mtb}$ acetylates a wide variety of N-terminal residues including hitherto unknown residues like Leu, Tyr, Glu, and Asp, *in vitro*. Finally, $RimI^{Mtb}$ is identified to exhibit relaxed acceptor substrate specificity and stringent donor specificity as opposed to eukaryotic NATs.

Further to define the substrate preference of $RimI^{Mtb}$ vis' a vis' eukaryotic NATs namely, NatA-NatF, custom synthesized peptides representing typical NAT substrates were utilized, where in $RimI^{Mtb}$ could efficiently acetylate NatA, NatC and NatE substrates.

Further, DTNB assay was employed to determine the specific activity of $RimI^{Mtb}$ against each of these typical NAT substrates. The assay revealed that specificity of $RimI^{Mtb}$ is closest to NatE as compared to other NATs and accordingly hydrophobic residue at N termini is the preferred substrate.

Subsequent homology modelling of $RimI^{Mtb}$ and its structural comparison with crystal structures of archaeal TvArd1 (specificity closer to NatA) and eukaryotic Naa50p (NatE) revealed conservation of key catalytic residues between the three proteins. The putative hydrophobic pocket of $RimI^{Mtb}$, identified in the model, further explains better stabilization of hydrophobic N-termini. Results of our *in vitro* experiments put together with *in silico* sequence/structure analysis finally suggest that the substrate specificity of $RimI^{Mtb}$ is closer to eukaryotic NatE than that of NatA. In *Mtb* at least 12% of reported Nα-acetylome bears Nα-acetylated Met at N-terminus. Therefore, $RimI^{Mtb}$ by its NatE like specificity could be the primary NAT responsible for acetylating such substrates in *M. tuberculosis*.

STRING database predicts interactions between $RimI^{Mtb}$ and its neighbourhood proteins. Similarly, Tuberculist suggest *Rv3423c-Rv3419c* as an operon. The genetic context of ORF *Rv3420c* is both conserved and essential. Therefore we explored enzyme–substrate interactions between $RimI^{Mtb}$ and its neighbourhood proteins. *In vitro* acetylation assays using mass spectrometry revealed acetylation of N-terminus of at least three neighbourhood important/ essential proteins of *M. tuberculosis* namely, chaperones: GroES, GroEL1, and homologs of conserved tRNA-A_{37}-t^6A modifying enzymes: TsaD. Protein-protein interactions between $RimI^{Mtb}$ and GroES, and $RimI^{Mtb}$ and TsaD were corroborated using Mycobacterial Protein Fragment Complementation Assays (MPFC), *in vivo*. However, through DTNB assay significant activity of $RimI^{Mtb}$ was observed only against GroES. GroES is identified as Nα-acetylated in Mtb proteome, previously. Therefore GroES is suggested as potential cellular substrate of $RimI^{Mtb}$.

Finally, to understand the physiological significance of $RimI^{Mtb}$ in mycobacteria, a knockout of $RimI^{Mtb}$ orthologue in *M. smegmatis* is created. A proof-of the concept comparative analysis of morphology, growth-kinetics and partial acetylome of knockout versus wild type strains indicate involvement of $RimI^{Mtb}$ in acetylation of cellular proteins and subtle effects on cellular growth rates under normal conditions. However, further comprehensive studies are required to understand physiological significance of $RimI^{Mtb}$ unambiguously, in mycobacteria.