

Depletion of the essential CCA-adding enzyme in *Mycobacterium tuberculosis* leads to selective reprogramming of the DNA damage response and a hyper-mutator phenotype.

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Supplementary data:

Supplementary Fig. 1: qRT-PCR analysis of *mtrA* gene in control and MtrA-depleted mutant strain.

Supplementary Fig.2: General schematic of the aTc depletion and drug/agent treatment protocols used in this study.

Supplementary Fig.3: Concordance between transcriptomic and proteomic responses.

Supplementary Fig. 4: Principal component analysis (PCA).

Supplementary Fig. 5: Global analysis of total RNAs isolated from wild-type and mutant *M. tuberculosis* strains.

Supplementary Fig. 6: Global analysis of DNA damage response of wild-type and mutant *M. tuberculosis*.

Supplementary Fig. 7: ShinyGO biological process enrichment analysis and dotplot charts.

Supplementary Fig.8: ShinyGO biological process enrichment analysis and dotplot charts.

Ad_Figure 1: Uncropped X-ray film showing the Western blot results presented in Figure 1.

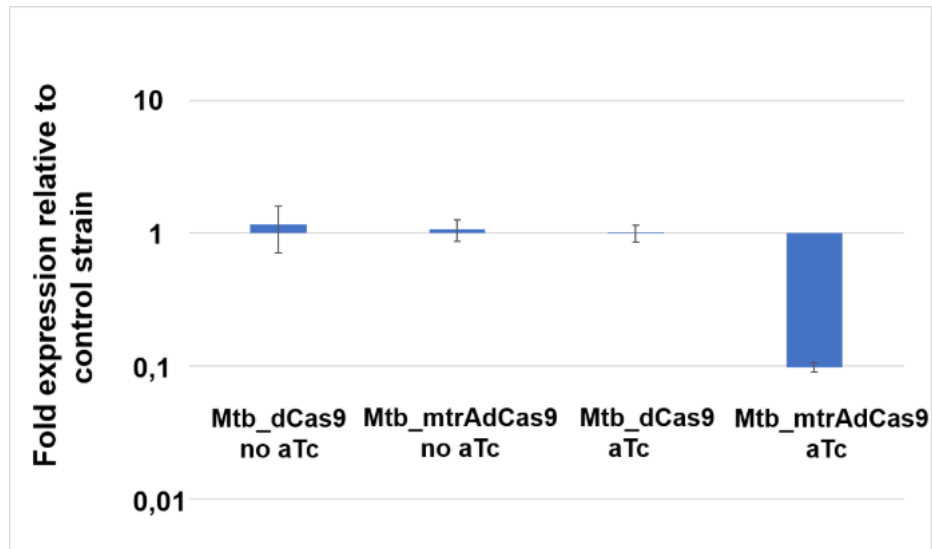


Figure 1. qRT-PCR analysis of *mtrA* gene in *M. tuberculosis* control strain and MtrA-depleted mutant. Mtb-Cas9 represents *M. tuberculosis* H37Rv carrying an empty dCas9 vector. Mtb-mtrAdCas9 represents a mutant with inducible depletion of MtrA. aTc represents supplementation of the culture with the inducer, 100 ug/mL of anhydrotetracycline. Expression was quantified by qRT-PCR using SYBR Green chemistry, normalized to the housekeeping gene *sigA*, and expressed as the mean $\pm$ SD from three independent experiments.

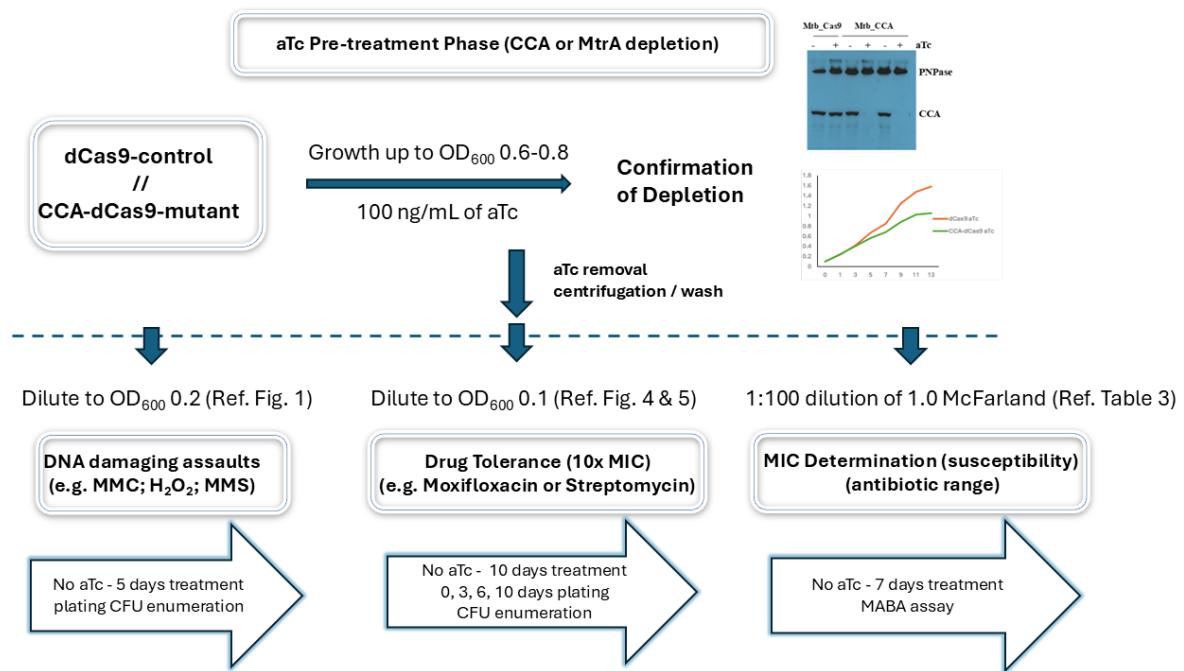


Figure 2: General schematic of the aTc depletion and drug/agent treatment protocols used in this study. The diagram outlines the standard vertical flow for all experiments involving the CRISPRi depletion of CCA or MtrA proteins. The top section illustrates the common "aTc Pre-treatment Phase," during which cultures are grown with 100 ng/mL anhydrotetracycline (aTc) to induce protein depletion until reaching target densities (typically OD₆₀₀ 0.6–0.8, though up to 1.0 for some tolerance uninduced controls). An inset illustrates illustrative confirmation methods (Western blot and typical growth kinetics of control vs. depleted strain). At the endpoint of this phase, aTc is removed, and cells are washed/diluted into fresh medium. The vertical arrows branch into three specific paths detailed in this study:

DNA-Damaging Assaults (Survival) (Ref. Fig. 1): Illustrates the protocol for genotoxicity assays (e.g., MMC, , MMS). The depleted culture is diluted to OD₆₀₀=0.2 in fresh medium (NO aTc) before agent addition. Incubation with the agent proceeds for 5 days without aTc before CFU plating.

Drug Tolerance (Survival) (Ref. Figs. 4 & 5): Details the timeline for 10-day tolerance assays. The depleted culture is diluted to OD₆₀₀=0.1 in fresh medium (NO aTc) before antibiotic addition at 10x MIC. Sampling occurs at days 0, 3, 6, and 10 for CFU enumeration.

MIC Determination (Susceptibility) (Ref. Table 3): outlines the MIC protocol. A standardized inoculum (e.g., from a McFarland standard) is diluted into fresh liquid medium (NO aTc) containing a range of antibiotic concentrations and incubated until growth controls indicate completion (visual MABA assessment, blue to pink color change).

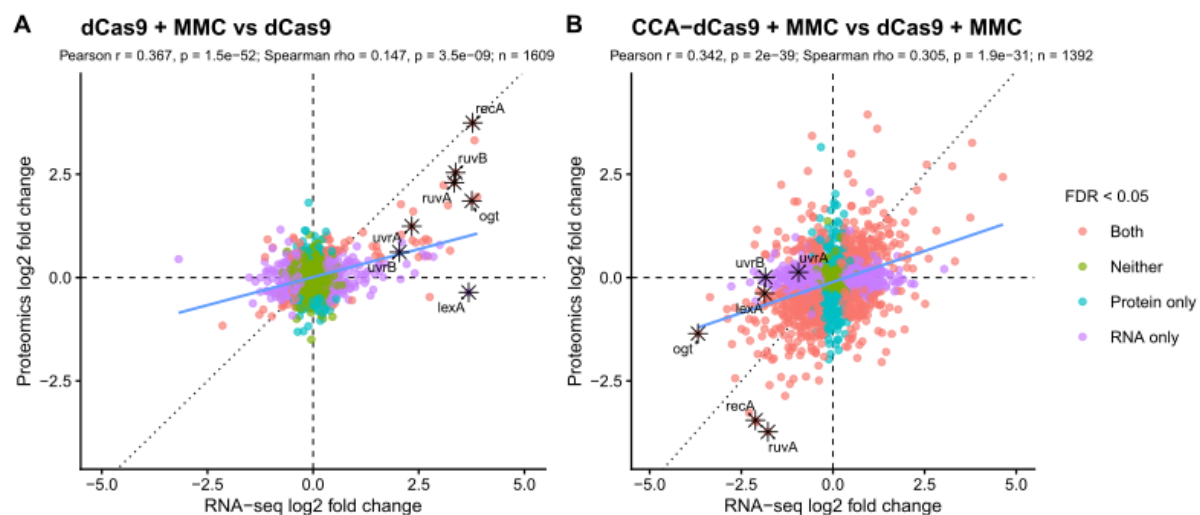


Figure 3: Concordance between transcriptomic and proteomic responses. RNA-seq and proteomic log2 fold changes were compared for genes/proteins matched by Rv locus tags. (A) dCas9 + MMC vs. dCas9 (n = 1,609; Pearson $r = 0.367$; Spearman $\rho = 0.147$). (B) CCA-dCas9 + MMC vs. dCas9 + MMC (n = 1,392; Pearson $r = 0.342$; Spearman $\rho = 0.305$). Selected components of the DNA damage response are indicated.

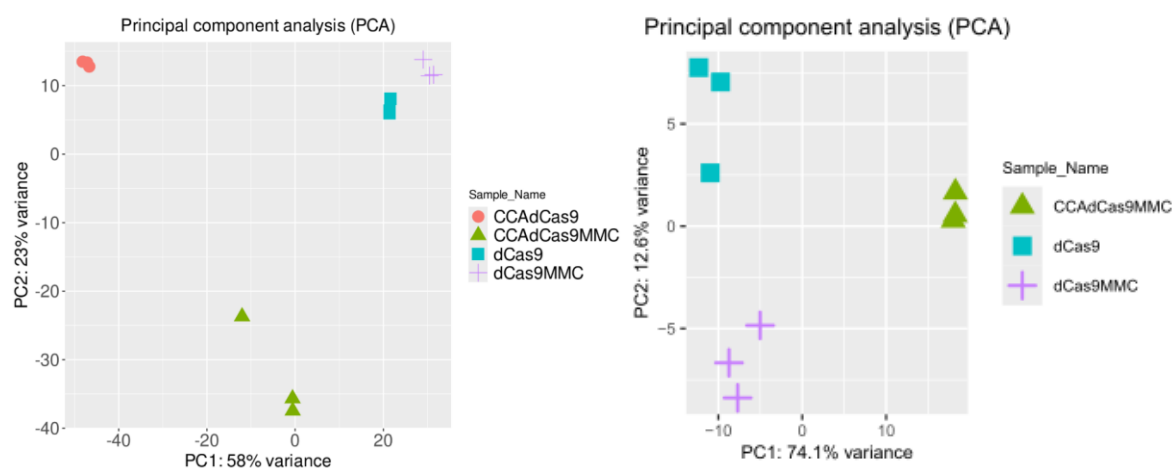
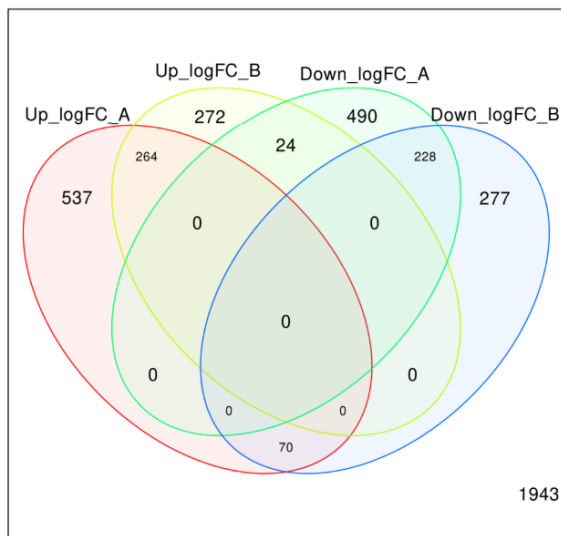
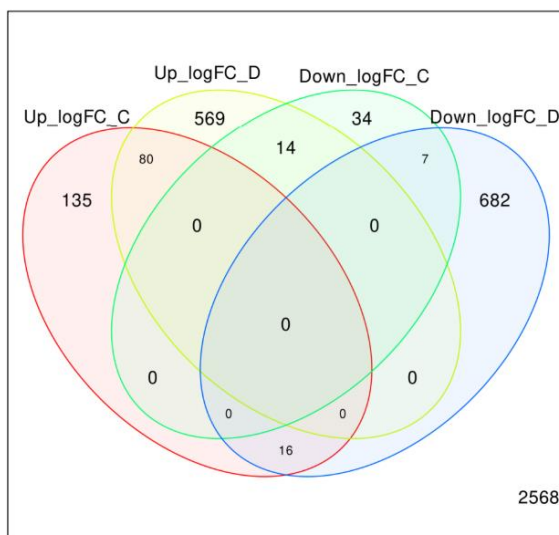


Figure 4: Principal component analysis (PCA) of the biological repeats of bacterial cultures growing for RNASeq analysis (left panel) and Proteomics (right panel). dCas9 represents control *M. tuberculosis* H37Rv strain carrying an empty dCas9 vector. CCAdCas9 represents *M. tuberculosis* H37Rv strain with inducible depletion of CCA enzyme. MMC represents supplementation of media with Mitomycin C. All cultures were growing up in media supplemented with 100 $\mu\text{g}/\text{mL}$ of aTc. Each repeat of the individual strain is marked by orange circle, green triangle, blue square, and purple cross. The analysis was completed using iDEP.96 platform (RNASeq) or RStudio (Proteomics). PCA of proteomic samples is based on log2-transformed MaxQuant LFQ intensities.



	UP	DOWN
A - dCas9 vs. CCA-dCas9	871	742
B - dCas9 MMC vs. CCA-dCas9 MMC	560	575

Figure 5: Global analysis of total RNAs isolated from wild-type and mutant *M. tuberculosis* strains. Venn diagram presenting differential gene expression of control *M. tuberculosis* H37Rv strain carrying an empty dCas9 vector (dCas9) and mutant, *M. tuberculosis* H37Rv strain with inducible depletion of CCA enzyme (CCA-dCas9). MMC represents supplementation of media with Mitomycin C. All RNASeq cultures were growing up in media supplemented with 100 µg/mL of aTc. The analysis was completed using iDEP.96 platform (FDR ≥ 0,1; logFC ≥ 2).



	UP	DOWN
C - dCas9 MMC vs dCas9	231	55
D - CCA-dCas9 MMC vs CCA-dCas9	663	705

Figure 6: Global analysis of DNA damage response of wild-type and mutant *M. tuberculosis*. Venn diagram presenting differential gene expression of control *M. tuberculosis* H37Rv strain carrying an empty dCas9 vector (dCas9) and mutant, *M. tuberculosis* H37Rv strain with inducible depletion of CCA enzyme (CCA-dCas9). MMC represents supplementation of media with Mitomycin C. All RNASeq cultures were growing up in media supplemented with 100 µg/mL of aTc. The analysis was completed using iDEP.96 platform (FDR ≥ 0,1; logFC ≥ 2).

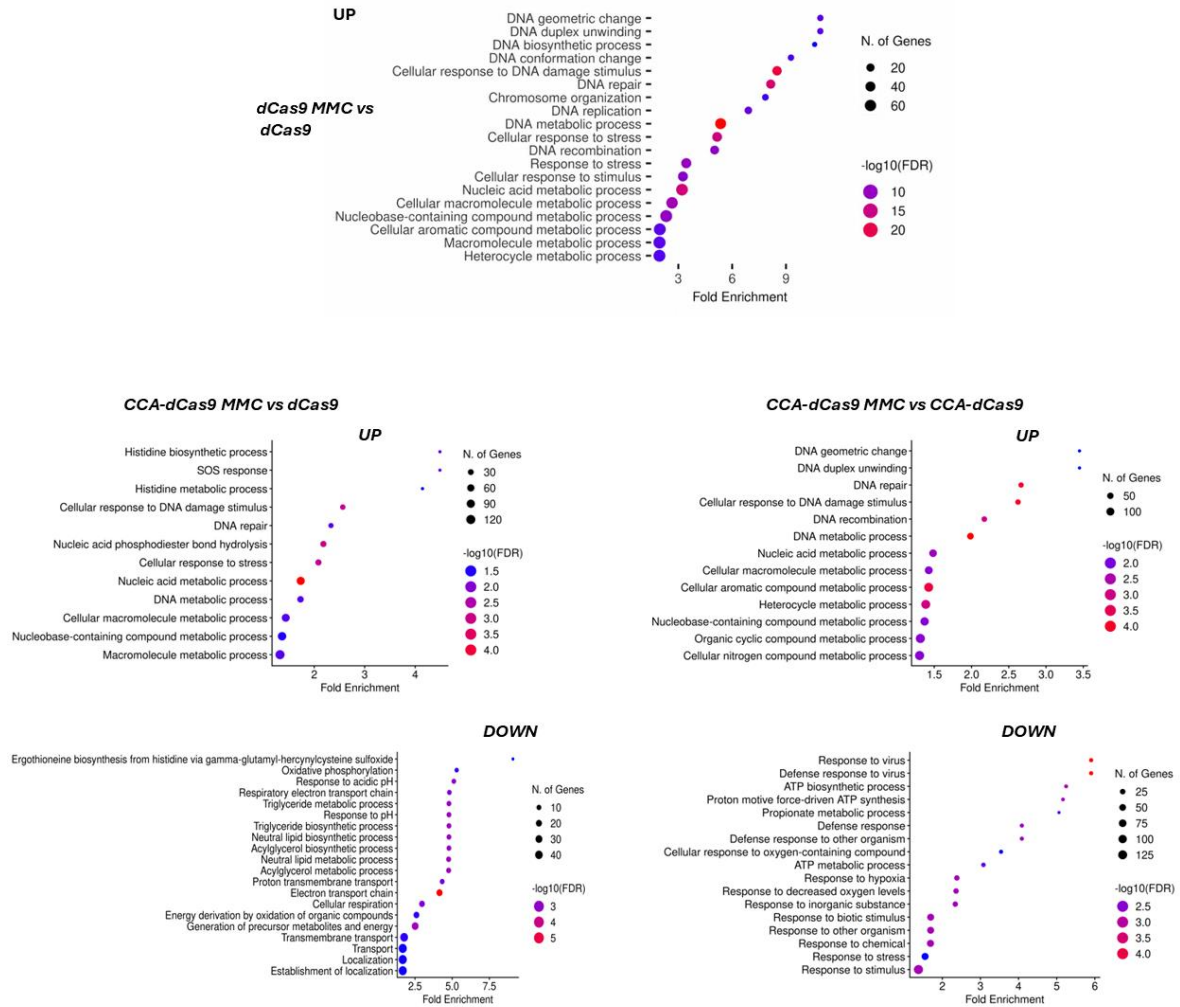


Figure 7: ShinyGO biological process enrichment analysis and dotplot charts. dCas9 represents control *M. tuberculosis* H37Rv strain carrying an empty dCas9 vector. CCA-dCas9 represents *M. tuberculosis* H37Rv strain with inducible depletion of CCA enzyme. UP (FDR ≤ 0.05 ; $\log_{2}FC \geq 1$) and DOWN (FDR ≤ 0.05 ; $\log_{2}FC \leq -1$) regulated genes. MMC represents supplementation of media with Mitomycin C. All RNASeq cultures were growing up in media supplemented with 100 $\mu\text{g/mL}$ of aTc.

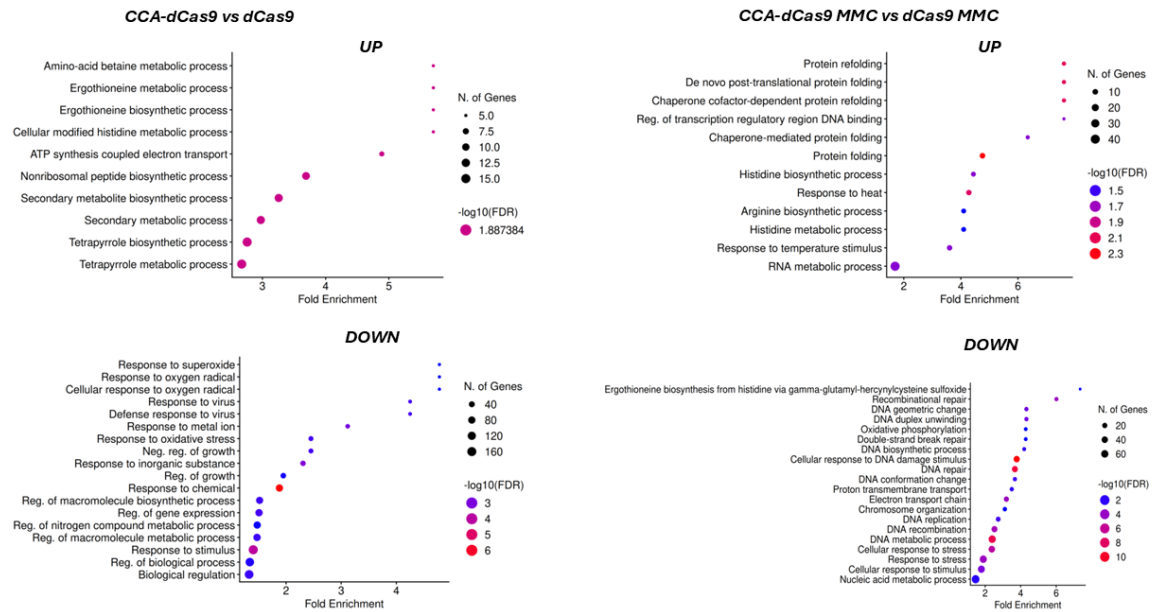
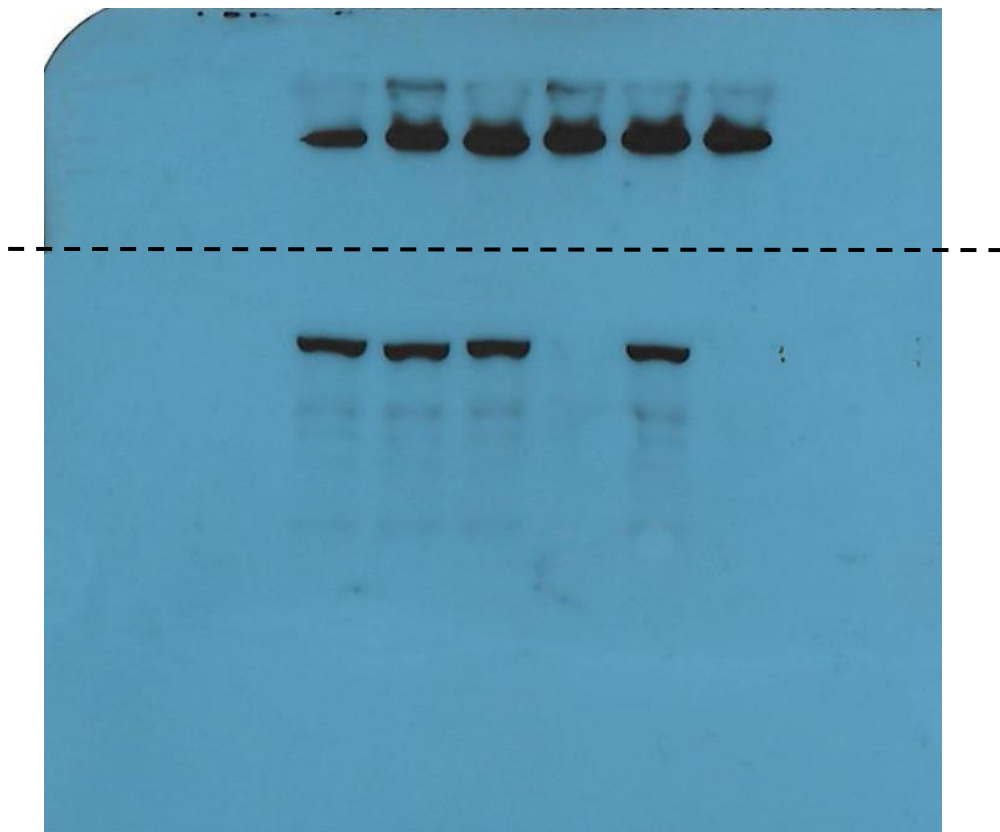


Figure 8: ShinyGO biological process enrichment analysis and dotplot charts. dCas9 represents control *M. tuberculosis* H37Rv strain carrying an empty dCas9 vector. CCA-dCas9 represents *M. tuberculosis* H37Rv strain with inducible depletion of CCA enzyme. UP and DOWN represent genes with log₂ fold change expression. UP (FDR ≤ 0.05; log₂FC ≥ 1) and DOWN (FDR ≤ 0.05; log₂FC ≤ -1) regulated genes. MMC represents supplementation of media with Mitomycin C. All RNASeq cultures were growing up in media supplemented with 100 µg/mL of aTc.



Ad_Figure 1: Uncropped X-ray film showing the Western blot results presented in Figure 1. Western blot analysis with anti-CCA and anti-PNPase polyclonal antibodies. Equal amounts of protein from each sample were separated via SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was divided into two sections, which were probed separately with polyclonal antibodies against CCA_{Mtb} (the target protein, 53.1 kDa) and PNPase (the loading control, 79.7 kDa) and co-exposed on a single X-ray film. The cut/assembly line of the blot is indicated by a dashed line.