CHARACTERIZATION OF NUDIX HYDROLASES: A UTILITARIAN
SUPERFAMILY OF ENZYMES

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Abstract

The present work details the structural and enzymatic characterization of Nudix hydrolases from three different organisms – *Bdellovibrio bacteriovorus*, *Mycobacterium tuberculosis*, and *Tetrahymena thermophila*. Each of these Nudix enzymes presents unique questions about their physiological function within their organism which are answered with a combination of structural biology, genetic manipulation, enzyme kinetics, and a wide range of protein assays.

We demonstrate that RenU, from *M. tuberculosis*, is part of Redox Homeostasis Control System (RHOCs), which senses and regulates NADH concentrations. This control system involves two other proteins, the serine/threonine protein kinase G (pknG) and the L13 ribosomal subunits, without which the bacterium fails to evade lysosomal delivery and falls prey to the oxidative arsenal of the macrophage host.

Bd-NDPSase, a Nudix enzyme encoded by the *B. bacteriovorus* gene BD3179, localizes to the periplasmic space of the bacterium and hydrolyses at least four nucleoside diphosphate sugars *in vitro*. Through atomic-resolution models from X-ray diffraction, we identified a motif that differentiates this hydrolase from the similar, but more substrate specific, ADP-ribose hydrolase from *E. coli*.

Lastly, we show that Nud1p from *T. thermophila* is a member of the Ezl1p complex, the histone methyltransferase Polycomb Group homologue of this protozoan. With the use of *in vitro* enzymatic assays we show, in addition, that Nu1dp hydrolyses CoA preferentially over acetyl-CoA and other nucleoside derivatives.
Advisor and first reader: Dr. Mario Amzel

Co-advisor and second reader: Dr. Sandra Gabelli
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The work on RenU would not have been possible without Drs. Liem Nguyen and Kirsten Wolff, who conducted the experiments in *Mycobacteria*. Likewise, the work on Nud1p would not have been possible without Drs. Sean Taverna and Romeo Papazyan, who conducted the experiments in *Tetrahymena*. I would like to thank Allison Suarez and Dr. Krisna Duong-ly for crystallizing Bd-NDPSase and for their critical review of the manuscript.
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CHAPTER 1

Introduction
1.1. The Nudix Hydrolase Superfamily

Since the discovery of the Nudix superfamily of hydrolases by Bessman in 1996 [1], these ubiquitous enzymes have been found in all domains of life. Originally characterized as “housecleaning” enzymes due to their ability to hydrolyze deleterious metabolites, Nudix enzymes are now known to, among many functions, gate ion channels [2, 3], decap mRNA [4, 5], and regulate redox homeostasis [6-8]. With the identification of their 23 amino acid signature motif, $G_1^N[5X]E_7^N[7X]R_{15}^NXXE_{19}^N E_{20}^N X G_{22}^N U$ (where U is I, L or V) [1], Nudix enzymes have been found in over 20,000 species [9]. Bessman named the Nudix superfamily to nucleate the proteins that contain this signature sequence since they shared the ability to bind divalent cations and hydrolyze substrates of the type nucleoside diphosphate linked to a moiety X (Nudix) [1]. While this sequence correctly identifies Nudix enzymes, functional and structural information is needed to fully classify the plethora of Nudix enzymes into families. For example, in *E. coli* alone, 13 Nudix enzymes corresponding to at least 10 families [10] have been identified. Likewise, in *H. sapiens* 24 enzymes from at least 14 families [10] and in *A. thaliana* 25 Nudix enzymes from at least 9 families [7] have been found.

1.2. The Nudix Fold

The Nudix signature sequence is found within a strand-loop-helix-loop structural element in an $\alpha$-β-$\alpha$ fold (Figure 1). The N-terminal helix of the fold ($\alpha$M) contains three glutamates ($E_{16}^N XXE_{19}^N E_{20}^N$) that are largely responsible for binding the divalent cations that bridge the protein to the pyrophosphate of the substrate. The mixed β-sheet of the fold contains 7 strands ($\beta_1 - \beta 7$) and is flanked at the C-terminus by two helices ($\alpha S_1$ and $\alpha S_2$). An arginine residue, located at the distal end of strand $\beta 2$ forms a bidentate hydrogen bond through the
guanidium to the pyrophosphate of the substrate and compensates the negative charge accumulation of the transition state. Helices αS1 and αS2 at the C-terminal of the fold generally contribute to substrate recognition through amino acid side chains at their termini (C-terminus and N-terminus, respectively).

**Figure 1. The Nudix Fold**
The Nudix signature sequence forms a strand-loop-helix-loop motif (cyan) and, together with loop LM, is responsible for metal binding. The N-terminal loop LN and loop LS (green) can accommodate a wide variety of extensions, insertions, and substitutions that contribute to structural and functional diversity. A mixed β-sheet (yellow) forms the core of the α-β-α Nudix fold. Helices αS1 and αS2 (grey) are found at the C-terminus and contribute to substrate recognition.

Although a complete picture of the structural determinants of substrate specificity has not emerged, insertions in loop LS, the addition of flanking domains, and quaternary
arrangements have been found to confer these enzymes the versatility to bind and hydrolyze a wide array of substrates. In other families, additional domains at either termini are also associated with more complex functions. For example, in the Nudix ADP-Rase [11], an antiparallel β-sheet domain proximal to LN is responsible for nucleoside recognition. In these dimers, the N-terminal domains and loops LS form extensive interactions with the opposite monomer and hydrogen bonding interactions with the substrates in both active sites. Also, in the multi-domain protein CCAR1 [12, 13], a SAP domain C-terminal to αS2 is believed to mediate DNA interactions. The versatility of Nudix enzymes is also observed in their catalytic mechanism. Often referred to as a mechanistically diverse superfamily [7], some families lack hydrolytic activity [14], while others have evolved to cleave their substrates in positions distal to the typical pyrophosphate scissile bond [15, 16]. These differences have been reconciled with observed changes in the signature sequence. For example, hydrophobic substitution at $E^{N}_{16}$ and $E^{N}_{19}$ ($G^{N}_{1}[5X]E^{N}_{7}[7X]R^{N}_{15}L^{N}_{16}XXA^{N}_{19}E^{N}_{20}XG^{N}_{22}U$) in nudD from *E. coli* results in a nucleophilic attack at carbon instead of the typical attack at phosphate [16].

### 1.3. Characterization

With enzymatic and bioinformatics studies, Dunn identified sequences elements downstream of the Nudix signature sequence that categorized Nudix enzymes into three families: the NADH hydrolases, the ADP-ribose hydrolases and the AP$_{6}$A hydrolases [17]. These sequence elements, 15 amino acids downstream of the Nudix sequence ($G^{N}_{22} + 15$), are in a loop joining strands β5 and β6. Since this loop is a determinant of substrate specificity, we call it specificity loop LS (Figure 1). Initially, a proline residue in this loop was identified as necessary for ADPR recognition in swapped dimers [17], but recently we
have shown that isoleucine can replace this proline. In the DHNTPase family, an insertion in this loop folds over the catalytic site and contributes to specificity [18]. In the cleavage factor Iₘ (CFIₘ) family, an elongated version of the loop LS interacts with its heterodimerization partner CFIₘ68 [19]. Another set of sequence elements is located 50 amino acids downstream of the Nudix sequence ($G_{22}^{N}+50$) in the loop joining strands $\beta 6$ and $\beta 7$. This loop generally contains one or two glutamate residues positioned to complete the coordination of the catalytic divalent cations together with $E_{16}^{N}, E_{19}^{N}$, and $E_{20}^{N}$. Since this loop binds a metal, we call it metal binding loop LM (Figure 1). Strand $\beta X$ represents the variable position where a helix or a loop is sometimes found.

Herein, we revisit Nudix enzymes whose in vitro substrate specificity has been elucidated in vitro and whose hypothetical function has been studied using a variety of in vivo experiments. We further characterize these enzymes into families based on substrate, amino acid sequence, and, when possible, atomic structure. In all, we have identified 3 Nudix families with functions as diverse as their structures. Some of these enzymes are dimeric, some contain tandem domains that confer specificity, while others borrow domains altogether to perform more complex functions.
CHAPTER 2

Bd-NDPSase, a nucleoside diphosphate sugar hydrolase

in *B. bacteriovorus*
*Bdellovibrio bacteriovorus* is a highly motile obligate predatory bacterium. It employs a large repertoire of hydrolases (the second largest density of hydrolases per genome [20, 21]) to infect, devour, and escape from other Gram-negative bacteria. There are no reports of mammalian cells being targeted by *Bdellovibrio bacteriovorus*, and its lack of type III and IV transport systems [21] makes it an ideal candidate for use as a live antimicrobial agent in humans. Furthermore, the large number of hydrolases and the precise timing of expression in its intricate biphasic life cycle can illuminate novel enzyme-based antimicrobial mechanisms and drug targets. Of particular interest is the *Bdellovibrio bacteriovorus* HD100 nucleoside diphosphate sugar (NDPS) hydrolase BD3179 [22] because it bears a striking structural homology to the adenosine diphosphate ribose (ADPR) hydrolase [11, 23] and guanosine diphosphate mannose (GDPM) hydrolase [24]. We expect that, its study may provide clues about the structural determinants of substrate specificity.

In light of recent findings, we propose to rename the GDPMase Nudix family as an NDPSase Nudix family, since these enzymes hydrolase at least three other NDPS analogues. The function of this family remains unknown. In *E. coli*, it was believed to regulate biofilm formation since it forms part of the RcsS regulon, but knockout studies did not show a significant effect on biofilm formation [24].

The ADPRase family has been extensively studied and is believed that its members regulate the ADPR pool and prevent non-enzymatic deleterious ribosylation [17, 23, 25]. Its function *in vivo* has not been elucidated.

Despite these distinct functions, both Nudix families share a high degree of structural homology that can be used to guide sequence-based identification of their substrate
specificity. For example, a proline 15 amino acids downstream of $G_{22}^N$ has been used to correctly predict substrate preference for ADPR [17]. However, Ec-NDPSase exhibits the same fold and $G_{22}^N+15$ pattern as the ADPRases, yet preferentially hydrolyzes two other nucleoside diphosphate sugars over ADPR [24]. By comparing the sequence elements in the substrate bound structures of *Escherichia coli* ADPRase [11] and NDPSase [24] we identified an aspartate-lysine sequence motif that correctly differentiates NDPSases from ADPRases.

### 2.1. *In vitro* studies

The *Bd3179* gene was amplified from *Bdellovibrio bacteriovorus* HD100 chromosomal DNA by PCR and cloned into the expression vector pET24a (Novagen, Madison WI). The encoded Nudix hydrolase, Bd-NDPSase, was expressed and purified to homogeneity. Its enzymatic activity was determined using a coupled enzyme colorimetric assay described in the section A1.1. Substrate specificity was investigated with a panel of several different nucleoside diphosphate derivatives (NDPX) and nucleoside triphosphates (NTP). Bd-NDPSase exhibited hydrolase activity with a substrate preference for nucleoside diphosphate sugars. Among the substrates tested, the wild type enzyme exhibited the highest relative activities for GDPM, UDPG, GDPG, and ADPR (Figure 2A, grey). Michaelis-Menten kinetic analysis revealed a $k_{cat}$ of 5200 s$^{-1}$ and a $K_m$ of 0.3 mM (Figure 2B) for the substrate with the highest relative hydrolysis, GDPM. This $K_m$ is comparable to those of other Nudix sugar hydrolases with a similar structure and molecular weight [17, 23-27]. Despite extensive crystallization trials, we were unable to obtain crystal structures of the binary or ternary complexes of Bd-NDPSase with GDPM, GDPG, or UDPG as intact substrates. Bd-NDPSase displayed much lower activities towards NTPs including ATP,
CTP, UTP, GTP, dATP, dCTP, and dUTP or other NDPXs such as AP₃P, AP₄P, UDP-GalNAc and UDP-GlcNAc. Substitution of the glutamate residue E140 (G₂₂ᴺ+5₀) in loop LM by a glutamine (E140Q), completely abolished the enzymatic activity of Bd-NDPSase (Figure 2A, red).

![Graph](image1)

**Figure 2. Bd-NDPSase wild type and mutant substrate specificity.**
A) Wild type enzyme (gray bars) exhibited preference for nucleoside diphosphate sugar (NDPS). E140Q mutant (red bars) were catalytically inactive. B) Initial rates of GDPM hydrolysis for the wild type and E140Q mutant were fit by nonlinear least squares to the Michaelis-Menten equation (solid lines) to determine $k_{cat}$ (5.2 (ms)$^{-1}$) and $K_m$ (0.3 mM). Standard deviations of triplicate measurements are shown by the shaded area for the wild type (gray shade) and mutant (red shade).

### 2.2. In vivo studies

#### 2.2.1. Cellular localization of *B. bacteriovorus* NDPSase

We determined the subcellular localization of the Bd-NDPSase by raising a polyclonal antibody against the purified enzyme. The primary antibody, visualized by transmission
electron microscopy (TEM) using a gold particle-conjugated secondary antibody, shows that Bd-NDPSase is localized in the periplasm of the cells (Figure 3).

Figure 3. Cellular localization of Bd-NDPSase.
A-B) TEM micrographs at 37,000x magnification showing the location of the secondary gold antibody (black dots) in the periplasmic space of a Bdellovibrio bacteriovorus cell. C) TEM micrographs at 20,000x magnification showing the location of the secondary gold antibody (solid, black spots) in the periplasmic space of multiple Bdellovibrio bacteriovorus cells.

Identification of a Nudix enzyme endogenous substrate has proved elusive largely due to the tendency of Nudix enzymes to hydrolyze more than one substrate in vitro. For instance, MutX hydrolyses all 8 canonical nucleotide triphosphates [28]. Furthermore, substrate specificity is often a function of the divalent cation used for in vitro studies. In the absence of a de facto standard to determine the metal cofactor in vivo, most Nudix studies have been conducted assuming that magnesium or manganese [29-34] is the endogenous cofactor and determining the best substrate based on their K_m and k_cat or their specificity constant k_cat/K_m. However, functional knockout studies have shown the preferred substrate in vitro to have no effect on the hypothesized phenotype. For example, Arabidopsis thaliana nudt7 [8] and Mycobacterium tuberculosis renU [6] functional knockouts did not show an effect on the in vivo concentration of the best in vitro substrate, ADPR. Instead, these knockouts showed an accumulation of NADH [6, 8], which, for the mycobacterial enzyme, showed a 10-fold lower specificity constant than ADPR [6]. The different
microenvironments in a cell have been shown to contain different concentrations of
divalent cations and Nudix substrates. We thus believe that the periplasmic localization of
Bd-NDPSase may be an important determinant of its substrate specificity.

2.3. Structural studies

2.3.1. Crystallization

Wild type and E140Q Bd-NDPSase crystals were grown by hanging drop vapor diffusion
at 20 °C. The drop, containing 1 μl of the reservoir solution (1.75-2.0 M ammonium sulfate,
0.1 M HEPES pH 7.0, and 0-0.5% PEG 8000) and 1 μl of 9.5-11.0 mg/ml of enzyme in 50
mM Tris-HCl pH 8.6, 1 mM EDTA, was equilibrated over a 1 mL of reservoir solution.
Crystals were derivatized with 5mM SmCl$_3$ for 48 hours, and either UO$_2$(ClO$_4$)$_2$ or 1 mM
GdCl$_3$ for 5 days in preparation for multiple isomorphous replacement. Crystals of the Bd-
NDPSase-ADPR binary complex were obtain by soaking crystals of the E140Q mutant in
5 mM ADPR for 2 days. Cocrystallization of Bd-NDPSase with 5mM UDPG did not give
the expected complex. Instead, the crystals contained the protein-glucose complex.
Data for the native crystal were collected at beam line X6A of the Brookhaven National
Laboratory, National Synchrotron Light Source. All heavy atom derivative data were
collected on a copper rotating anode x-ray generator (RU-H3R) as the X-ray source and an
RAXIS IV (Rigaku) as the detector, at the X-ray facility of the Department of Biophysics
and Biophysical Chemistry of the Johns Hopkins University School of Medicine. Indexing
and data reduction were carried out with the HKL2000 suite[35]. Phases were calculated
using the SmCl$_3$, UO$_2$(ClO$_4$)$_2$ and GdCl$_3$ derivatives with the program SOLVE[36, 37].
Density modification and an automatic partial model were built using the program
RESOLVE[38, 39]. The final model was built and refined using iterative cycles of manual
building and maximum likelihood refinement with the programs Coot and REFMAC5 of the CCP4 suite [40-43]. Data statistics are shown in Table 1.

Table 1. Bd-NDPSase crystallographic data collection and refinement statistics.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Wild type</th>
<th>E140Q Glucose</th>
<th>ADPR</th>
<th>Wild type</th>
<th>Wild type</th>
<th>Wild type</th>
</tr>
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<tr>
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<td>P2₁,2,2</td>
<td>P2₁,2,2</td>
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<tr>
<td>Cell dimensions</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a, b, c (Å)</td>
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<td>75.9, 103.3, 51.7</td>
<td>75.6, 103.4, 51.6</td>
<td>75.8, 103.4, 51.8</td>
<td>76.3, 103.0, 51.7</td>
<td>76.1, 103.5, 52.0</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
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<td>90, 90, 90</td>
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<td>90, 90, 90</td>
<td>90, 90, 90</td>
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<tr>
<td>Resolution ( Å )</td>
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<td>50.00-2.06</td>
<td>50.00-2.43</td>
<td>50.00-2.52</td>
<td>50.00-1.95</td>
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<tr>
<td>R_{symm} (%)</td>
<td>11.1 (63.6)</td>
<td>6.4 (46.6)</td>
<td>9.5 (52.5)</td>
<td>11.6 (45.9)</td>
<td>11.5 (41.6)</td>
<td>7.7 (48.0)</td>
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<td>I/σ</td>
<td>23.9 (1.38)</td>
<td>42.8 (3.04)</td>
<td>17.6 (2.3)</td>
<td>23.6 (3.5)</td>
<td>27.8 (4.6)</td>
<td>35.7 (2.5)</td>
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<tr>
<td>Completeness (%)</td>
<td>94.5 (80.9)</td>
<td>92.4 (54.1)</td>
<td>95.0 (68.9)</td>
<td>99.9 (99.9)</td>
<td>95.4 (95.1)</td>
<td>90.8 (84.9)</td>
</tr>
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<td>Redundancy</td>
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<td>5.7 (3.4)</td>
<td>5.4 (2.1)</td>
<td>5.9</td>
<td>5.8</td>
<td>5.7</td>
</tr>
</tbody>
</table>

The ligand-bound structures were built in a Fourier map using phases of the wild type protein. The initial model was rebuilt in Coot [42] and refined using REFMAC5 [44]. Quality of the structures was assessed with Coot [42] and online validation tools from the PDB [45]. Figures were drawn with PyMOL [46].
Atomic coordinates and structure factors of the wild type Bd-NDPSase (PDB ID 5C7Q), Bd-NDPSase E140Q in complex with ADP-ribose (PDB ID 5C7T), and Bd-NDPSase E140Q in complex with glucose (PDB ID 5C8L) were deposited in the Protein Data Bank.

### 2.3.2. Overall structure

Bd-NDPSase is a homodimer whose monomers are related by a non-crystallographic 2-fold axis. Each monomer comprises an N-terminal β-sheet and a Nudix domain (Figure 4). The N-terminal domain contains three anti-parallel strands (residues 1-40) connected by loops. Loop L-2 connects strands β-3 and β-2 and contributes to a π-stacking interaction with the substrate via Y19. The C-terminal domain, comprising the Nudix fold, is composed of an α-β-α motif (αM, β1-β7, αS). The specificity loop LS joining strands β5 and β6 of the mixed beta sheet interacts with the substrate in the catalytic site of the opposite monomer via hydrogen bonding. The swapped N-terminal domains and the Nudix fold form two catalytic cavities with an exposed surface of ~689 Å² each. The signature Nudix sequence is folded as loop-helix-loop (β4-L3-αM-L4). Its conserved glutamates $E_{16}^N, E_{19}^N, E_{20}^N$, together with E140 of loop LM, are positioned to coordinate Mg²⁺ binding.
Figure 4. Overall Structure Bd-NDPSase.

A) Structure of E140Q Bd-NDPSase bound to ADPR (PDB ID 5C7T). B) Sequence alignment of the Ec-ADPRase (PDB ID 1KHZ) and Ec-NDPSase (PDB ID 3O61) to Bb-NDPSase. The N-terminal domain (residues 1-44, green) consists of an antiparallel beta sheet (β-3 to β-1, yellow). The Nudix fold consists of a mixed beta sheet (β1-β7) flanked by helix αM on one side (cyan) and helices αS1 and αS2 (residues 148-182, gray) on the other side. The location of the E140Q mutation is denoted by an asterisk.
An extensive dimer interface (2,992 Å²) spans the largest axial cross-section of the homodimer. The dimer interface spans both N-terminal and C-terminal domains and separates the Nudix folds of each monomer along the non-crystallography 2-fold axis. It includes interactions between domain homologous elements as well as domain swapped elements (Figure 5). Not surprisingly, substrate binding involves elements from both monomers. In fact, 46% of the buried ADPR surface in each active site is from the opposite monomer.

**Figure 5. Bd-NDPSase dimer interface.**
The dimer interface was calculated using the PISA web server [47]. Structural elements are colored as in Figure 4. The side chains involved in dimer contacts are represented by red ribbons (A and C) and a red surface (B and D). Panels C-D are viewed at a 90° rotation from A-B.
2.3.3. ADPR recognition

Adenosine recognition involves hydrogen bonding by the E38’ (prime denotes a residue of the other monomer) main chain amide nitrogen and oxygen to the adenosyl N1 and N6 respectively. The main chain oxygen of G115’ bridges the adenosyl N6 and N7. R37’ and Y19 flank the nucleoside base though stacking interactions on opposite sides (Figure 6 and Figure 7A). Analogous stacking interactions, involving the conserved arginine on strand β-1 and the conserved aromatic residue on loop L-2, are also present in Ec-NDPSase and Ec-ADPRase (Figure 7).

Figure 6. Recognition of ADPR by Bd-NDPSase.
A) Ribbon representation of the catalytic site of the ADPR-bound Bd-NDPSase crystal structure (PDB ID 5C7T). B) Schematic representation of the recognition of ADPR by Bd-NDPSase. Catalytic helix (αM) residues are shown in cyan, catalytic loop LM residues are shown in magenta. N-terminal domain residues (1-44) are shown in green as is the specificity loop LS. Hydrogen bonds are shown as orange dashes. The prime symbol (’) denotes residues of the opposite monomer.
In all cases, the nucleoside is stacked by an aromatic residue in loop L-2 and an arginine in strand β-1’. A) Adenosine recognition by the Bd-NDPSase hydrolase (PDB ID 5C7T). B) Guanosine recognition by the Ec-NDPSase hydrolase (PDB ID 3O61). C) Adenosine recognition by the Ec-ADPR hydrolase (PDB ID 1KHZ). Substrate carbons are shown in black, residue carbons are colored using the main chain color convention. Nitrogen and Oxygen are colored in blue and red respectively. Hydrogen bonds are shown as orange dashes. The prime symbol (’) denotes elements of the opposite monomer.

Comparison of the ADPR-bound structure with the apo structure (PDB IDs 5C7T and 5C7Q) shows that neither of these residues are displaced upon substrate binding, suggesting that a gating product-release mechanism, as described by Yoshiba [27], may not involve these residues in Bd-NDPSase. A product-release mechanism, like the one described by Boto [24], could involve an E140-Mg$^{2+}$ mediated loop LM gating of the substrate in the Mg$^{2+}$-ADPR ternary complex. The B-factor of the E140 Ca in apo Bd-NDPSase is 3.1 times larger than that of G115’ on loop LS, and 1.7 times larger than that of Y19 on loop L-2, suggesting that loop LM’s intrinsic flexibility facilitates substrate binding followed by E140 mediated nucleophilic attack of the scissile phosphate. The proposition that E140 functions in catalysis is strengthened by the fact that substitution by glutamine rendered the enzyme catalytically inactive, as evidenced by both relative activity assays and kinetic assays (Figure 2). Another plausible mechanism employing LM displacement is that proposed by Nakamura [48] in which a third cation neutralizes the
negative charge buildup of the transition state concomitantly with the displacement of an aspartate residue.

As in Ec-NDPSase [24], Ec-ADPRase [11], and Mt-ADPRase [23], R64 of Bd-NDPSase forms a bidentate hydrogen bond through the guanidinium side chain with the β-phosphate oxygens O1β and O2β (Figure 8). It should be noted that this mode of binding is present in metal-substrate bound ternary structures as well as substrate bound binary structures [11, 24]. This suggests that, in addition to serving as a Lewis acid to facilitate release of ribose phosphate [49], R64 recognizes and orients the β-phosphate of the substrate. Mutational studies in a homologous Nudix hydrolase suggest that R64 is involved in both substrate binding and catalysis as evidenced by an increase in $K_m$ and a decrease in $k_{cat}$ upon arginine substitution by glutamate[16]. Comparison of the substrate bound Ec-NDPSase to the Ec-ADPRase reveals that the highly conserved arginine on strand β2 (Bd-NDPSase R64) is ideally positioned for charge compensation of the sugar-phosphate product (Figure 6 and Figure 8A).

![Sugar recognition by bacterial Nudix sugar hydrolases.](image)

**Figure 8. Sugar recognition by bacterial Nudix sugar hydrolases.**

A) Ribose recognition by the Bd-NDPSase (PDB ID 5C7T). B) Mannose recognition by the NDPS hydrolase (PDB ID 3O61). C) Ribose recognition by the ADPR hydrolase (PDB ID 1KHZ). Substrate carbons are shown in black, residue carbons are colored using the main chain color convention. Nitrogen and Oxygen are colored in blue and red respectively. Hydrogen bonds are shown as orange dashes. The prime symbol (’) denotes residues of the opposite monomer.
In addition to nucleoside and phosphate recognition, Bd-NDPSase forms a network of hydrogen bonding interactions involving the three hydroxyl groups of the distal ribose. G115' tethers both ends of an ADPR horseshoe conformation by hydrogen bonding to the hydroxyl at C2 through its main chain amide nitrogen as well as to adenosine through the amide oxygen. The carboxylate of E119 makes a hydrogen bond with the hydroxyl at C3 and positions a water molecule to make a hydrogen bond to the hydroxyl on C2 (Figure 8). The most distinguishing amino acid residues are K165 and D163, which form a bridge between the hydroxyl at C1 and the carbonyl oxygen at the Cβ of T166. Interestingly, this network of interactions is also present in Ec-NDPSase, but not in Ec-ADPRase, suggesting that these residues may indeed be responsible for differentiation between GDPM and ADPR[24].

2.3.4. Glucose recognition

Bd-NDPSase crystals soaked with UDPG displayed electron density that was interpreted and refined as a glucose molecule in each active site of the asymmetric unit. Modeling of glucose, sulfate, and glycerol molecules in the unbiased electron density revealed that these molecules are located within hydrogen bonding distance of catalytic residues (Figure 9). R64 makes a hydrogen bond with the sulfate and glucose molecules through its guanidinium side chain. Water molecules mediate the interactions of these molecules with other key residues such as E96, Q140, and K165. The glucose molecule is shifted toward the expected position of the phosphate of a phosphohexose product (Figure 9).
Figure 9. Glucose recognition by Bd-NDPSase.
Substrate carbons are shown in black, residue carbons are colored using the main chain color convention. Nitrogen and Oxygen are colored in blue and red respectively. Hydrogen bonds are shown as orange dashes.

2.3.5. Modularity of Bd-NDPSase

The phylogenetically conserved Nudix enzymes have been tuned to perform one catalytic activity, the hydrolysis of a diphosphate or a phosphate ester, and evolutionarily repurposed to perform diverse functions in the cell. Their versatility arises from a modular design where substrate recognition is independent from substrate hydrolysis. In fact, several Nudix proteins have been characterized as proteins that bind, but do not hydrolyze substrates [2]. The structure of the dimeric Bd-NDPSase, reveals that each monomer can be subdivided into three regions with exclusive roles: 1) the Nudix fold (Figure 1: magenta, cyan, yellow, gray) composed of the α-β-α motif (αM, β1-β7, αS1,2); 2) the nucleoside recognition module (Figure 1: green) composed of L-2, LS’ and β-1’ of the N-terminal domain; and 3) the sugar recognition module (Figure 1: green, gray) composed of LS, LS’ and αS1,2 of the
Nudix fold. The Nudix fold can be subdivided into three modules with different functions: a *metal binding* module formed by the glutamates of the Nudix signature sequence (Figure 1: cyan); a *phosphate charge compensation* module formed by amino acids in β2 (Figure 1: yellow); and a *catalytic base gating* module formed by loop LM (\(\theta_{22}^{N} + 50\); Figure 1: magenta). Changes in each of these modules repurpose the enzyme for a specific physiological function. Importantly, they could also be used to target these enzymes in the laboratory for additional functions.

### 2.3.6. Differentiation of ADPRases and NDPSases

Despite the low degree of sequence identity and similarity (13.7% and 16.4%) of the *E. coli* ADPRase and NDPSase to Bd-NDPSase there is a high degree of homology in their structures as well as in their quaternary arrangement, as shown by low RMSD 1.34 and 1.4 Å respectively (Figure 10). Sequence identity (22 of 26 residues) and similarity (26 of 30 residues) are accounted for mostly by the Nudix fold. The N-terminal domains contain four identical residues and four similar residues. Of these residues, only Y19, R37’, and E38’ mediate nucleoside recognition (Figure 7A). It is worthwhile noting that the mechanism for nucleoside recognition is conserved across all three proteins. The hydrophobic residue in loop L-2 stacks against the nucleosidic base while the tandem arginine and glutamate of strand β-1 mediate adenosine or guanosine recognition (Figure 7). The mechanism for recognition of the β-phosphate is likewise conserved in all three proteins, with the arginine at the end of strand β2 forming hydrogen bonds through its guanidinium side chain to the β-phosphate oxygens O1β and O2β. Therefore, differentiation between the substrates ADPR and GDPM must take place through a mechanism involving the derivative ribose and mannose as suggested by Boto[24]. The
present work supports this hypothesis since the same hydrogen bond network, involving
the aspartate and lysine at the N-terminus of helix $\alpha S_2$, is conserved and bound to substrate
only in Bd-NDPSase and Ec-NDPSase.

**Figure 10. Structures of three domain-swapped dimeric Nudix sugar hydrolases.**
Ribbon representation in which one monomer is colored in a lighter shade. A) Bd-NDPSase

ADPRases have been previously identified through sequence elements alone. The
ADPRases in *Haemophilus influenza* and *Bacillus subtilis* were correctly identified by
Dunn[17] using a proline 15 amino acids downstream from the C-terminal glycine of the
signature Nudix motif ($G_{22}^{N}+15$). Likewise, Boto [24] hypothesized that an aspartate-lysine
(DK) motif at the N-terminus of helix $\alpha3$ in Ec-NDPSase would correctly predict substrate
preference for GDPM over ADPR. This aspartate-lysine pair ($G_{22}^N+65$), in fact, correctly predicted that Bd-NDPSase prefers GDPM over ADPR. Furthermore, the highly conserved Proline ($G_{22}^N+15$) is not a necessary condition for NDPSase characterization as Bd-NDPSase possesses an isoleucine at this position. Both Ec-NDPSase and Bd-NDPSases also hydrolyzed UDPG preferentially over ADPR. We thus propose that this type of enzymes, characterized by a domain swapped N-terminal dimer, a signature Nudix motif in each molecule, and a DK motif on helix α3 be known as nucleoside diphosphate sugar hydrolase, “NDPSase.”

While the Nudix fold is largely responsible for recognition of the derivative sugar and diphosphate, the N-terminal domain recognizes the nucleosidic base. The highly conserved E38’ of Bd-NDPSase β3 forms a hydrogen bond with adenosine N5 and N6 through the main chain amide nitrogen and oxygen, respectively (Figure 7 A and C). As evidenced by the GDPM bound structure of Ec-NDPSase, for guanosine recognition (Figure 7B) to take place, it needs only to be displaced ~2.5Å from the adenosine position (Figure 7 A and C). This displacement places the guanosine within distance to form a hydrogen bond with the main chain amide nitrogen of the highly conserved glutamate in β3 and the main chain amide oxygen of the residue two positions upstream (Figure 7B). This mechanism explains how the NDPSases can recognize multiple nucleosides and, with the addition of the DK motif at α3, differentiate between the mannose and ribose derivative sugars.
CHAPTER 3

RenU, a nicotinamide adenine dinucleotide hydrolase in

*M. tuberculosis*

Material from this chapter has been abstracted from publication: Wolff KA, de la Peña AH, *et al.* A redox regulatory system critical for mycobacterial survival in macrophages and biofilm development. *PLOS Pathogens.* 2015;11(4):e1004839. doi: 10.1371/journal.ppat.1004839.
Despite a constant decrease in tuberculosis (TB) incidence rates, one-third of the world’s population has latent TB [50]. Five to ten percent of people with latent TB will develop the active form of the disease[51]. A critical determinant defining pathogenicity of *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis, is its survival in host macrophages. Upon internalization by the host phagocytic cell, *Mtb* and related pathogenic mycobacteria block the fusion of their resident phagosome to the destructive lysosome, thereby establishing a niche within the bactericidal macrophage [52, 53]. This ability of pathogenic mycobacteria requires the eukaryotic-type serine/threonine protein kinase G (PknG) [54]. Lack of PknG activity results in rapid delivery of mycobacteria to lysosomes, leading to enhanced killing of the intracellular bacilli by macrophages [54]. Besides its role in the innate survival of *Mtb* in host cells, PknG provides mycobacterial species with an intrinsic resistance to antibiotics [55]. In the absence of PknG, both pathogenic *Mtb* and non-pathogenic *M. smegmatis* display increased susceptibility to multiple antibiotics [55]. These observations suggest that PknG might be required for the persistence of *Mtb* during host infection, in which the bacillus also becomes highly recalcitrant to antibiotics. Although PknG represents an attractive target for tuberculosis (TB) drug development, the molecular mechanism by which this kinase exerts its biological functions remains largely unknown. It was shown that PknG is secreted via the SecA2 secretion system [56] into the macrophages’ cytosol [54] where, it is hypothesized, to interfere with host signaling pathways controlling phagolysosome synthesis [54]. However, attempts to identify the putative host substrate(s) targeted by PknG have thus far been unsuccessful. As a result, the role of PknG in *Mtb* survival in host macrophages remains ambiguous.
Here, we found that in mycobacteria host persistence is regulated by RHOCs, a newly identified Redox Homeostatic Control System in which PknG plays a central role. We show that the redox regulatory molecule NADH induces the expression of PknG, which phosphorylates the ribosomal protein L13 at a unique residue, threonine 11 (T11). The phosphorylation promotes the cytoplasmic association of L13 with RenU, a Nudix hydrolase encoded by a gene adjacent to pknG on mycobacterial chromosomes, and accelerates RenU’s NADH hydrolytic activity. Disruption of the PknG-L13-RenU pathway causes: (i) increased oxidative stress susceptibility, (ii) accumulation of NADH and FAD during oxidative stress, (iii) increased lysosomal delivery, and (iv) reduced survival of Mtb in host macrophages.

3.1. In vitro studies

To test whether RenU is indeed a hydrolase, and to further characterize its enzymatic activity, the recombinant M. smegmatis RenU was purified to homogeneity. Its solution oligomerization state (monomer) and purity were assessed by gel filtration chromatography (Figure 11).
Figure 11. Gel filtration chromatography indicates RenU is a monomer in solution. Gel filtration chromatography on a Sephacryl 16/60 S-200 column. Shown are elution profiles of RenU.6H (black, ~17 kDa) and two chromatography standards: cytochrome C (red, 12.4 kDa) and carbonic anhydrase (blue, 29 kDa).

RenU’s enzymatic activity was determined using a coupled enzyme colorimetric assay described in the section A1.1. Substrate specificity was investigated with a panel of several different nucleoside diphosphate derivatives (NDPX) and nucleoside triphosphates (NTP). RenU did exhibit Nudix hydrolase activity with a substrate preference for NDPXs. Among the substrates tested, the highest activities were observed with ADP-ribose, FAD, and NADH (Figure 12). By contrast, the enzyme displayed much lower activities towards NTPs including ATP, 7,8-dihydroneopterin triphosphate (DHNTP), dGTP, dCTP, dUTP, or other NDPXs such as CoA, GDP-D-mannose, NADP, ADP-ADP, and CDP-choline. Importantly, mutations in glutamate residues $E_{16}^N$, $E_{19}^N$, and $E_{20}^N$ (E74, E77, and E78) of the
Nudix box in αM, which are expected to coordinate the magnesium required for the activities of Nudix hydrolases, completely abolished the enzymatic activity of RenU. The mutated protein, RenU E(74,77,78)A, displayed no activity towards the preferred substrates exhibited by wild type RenU (Figure 12, red vs grey).

Figure 12. RenU relative Nudix hydrolase activity.
Wild type RenU (grey bars) exhibits the highest rates of *in vitro* hydrolysis with the NDPS substrates ADPR, FAD, and NADH. The RenU E(74,77,78)A mutant (red bars) exhibits no hydrolase activity towards the preferred substrates.

Michaelis-Menten analysis revealed that, *in vitro*, ADP-ribose and FAD were better substrates than NADH, as evidenced by their higher $k_{cat}/K_m$ value (Table 2). However, previous studies with Nudix hydrolases suggest that the substrate preference of RenU might be defined *in vivo* through its interactions with other proteins [57].

<table>
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<tr>
<th>Table 2. Kinetic parameters of RenU substrate hydrolysis</th>
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<td>Substrate</td>
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<td>NADH</td>
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<td>FAD</td>
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<td>ADPR</td>
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28
3.2. *In vivo* studies (Mycobacteria experiments conducted by KA Wolff)

3.2.1. Both pknG and its neighboring Nudix hydrolase gene renU are involved in redox homeostasis

Structural studies revealed a rubredoxin-like domain at the N-terminus of PknG, suggesting a possible involvement of this kinase in redox homeostasis [58]. We studied the role of the pknG locus in mycobacterial redox homeostatic control. Interestingly, these studies revealed that pknG and neighboring gene (*msmeg_0790/rv0413*) were each required for oxidative stress resistance in *M. smegmatis* and *Mtbc* (Figure 13). *msmeg_0790/rv0413* had been previously annotated as *mutT3* since the deduced amino acid sequence contains the Nudix motif. However, recent studies showed that *msmeg_0790* and *rv0413* are not involved in anti-mutation activity [59]. In light of the fact that this protein does not function as an anti-mutator, and the findings described in this paper, we propose to rename this Nudix hydrolase as RenU (for Redox Nudix hydrolase).
Figure 13. renU and pknG are required for *Mycobacteria* growth during oxidative stress

**A)** Both *pknG* and *renU* are each required for *M. smegmatis* resistance to H₂O₂ (left) and diamide (right). Wild type *M. smegmatis* (circles), *MsΔpknG* (triangles) and *MsΔrenU* (squares) were grown in 7H9 medium. At the indicated times (arrows), 10mM H₂O₂ or 15mM diamide was added. Growth was estimated through optical absorbance at 600 nm (OD₆₀₀nm). Error bars represent standard deviation of biological triplicates.  

**B)** *pknG* and *renU* are each required for *Mtb* resistance to H₂O₂ (left) and diamide (right). Wild type *Mtb* (circles), *MtbΔpknG* (triangles) and *MtbΔrenU* (squares) were grown in 7H9-OADC medium. At the indicated times (arrows), 20 mM H₂O₂ or 10 mM diamide was added. Growth was estimated through measuring optical absorbance at 600 nm.
3.2.2. NADH induces PknG expression

A recent study showed that expression of PknG is tightly regulated by unknown mechanisms related to the pathogenicity of \textit{Mtb} [60]. Whereas PknG is highly expressed in slow growing mycobacteria such as \textit{Mtb} and \textit{M. bovis} BCG, the expression in \textit{M. smegmatis} is extremely low [55, 60]. To investigate the conditions that trigger PknG expression in \textit{M. smegmatis}, the bacterium was treated with various redox stimuli, followed by analysis of PknG levels by Western analysis using a specific polyclonal antibody [54, 55, 60]. Interestingly, we found that PknG expression is uniquely induced when \textit{M. smegmatis} cells are exposed to high levels of NADH (Figure 14).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{induction_of_pknG_expression.png}
\caption{Induction of PknG expression in \textit{M. smegmatis}. Western analysis was used to detect PknG expression following the exposure of wild type \textit{M. smegmatis} cultures (OD600 of 2) to various oxidative stimuli including NADH (upper) and FAD (lower) for 30 minutes. All chemicals were used at 10 mM except for bleomycin, which was used at 10 μg/ml. Samples were separated by SDS-PAGE.}
\end{figure}
The induction of PknG expression by NADH, in both concentration (Figure 15A) and time-dependent (Figure 15B) manners, may suggest a specific regulatory mechanism, similar to the Rex system originally described in *Streptomyces* [61-64], or an indirect effect due to changes in cellular metabolism or physiology caused by NADH exposure.

**Figure 15. Effect of NADH on PknG expression**

**A)** Titration of the induced PknG expression by increasing NADH concentrations (0–30 mM) for 30 minutes. Samples were separated on SDS-PAGE, followed by immunodetection using an anti-PknG antibody or an anti-DivIVA antibody, as a control.

**B)** Time course of PknG expression (0–60 minutes) following cell exposure to 10 mM NADH. Samples were separated on SDS-PAGE, followed by immunodetection using an anti-PknG antibody or an anti-DivIVA antibody, as a control, followed by immunodetection using an anti-PknG antibody or an anti-DivIVA antibody, as a control. Non-induced lysates from wild type M. smegmatis and MsΔpknG were used as controls.

The facts that (i) PknG expression is induced by NADH (Figure 14 and Figure 15), (ii) RenU preferentially degrades this redox cofactor *in vitro* (Figure 12 and Figure 16), and (iii) absence of PknG or RenU leads to failed oxidative stress responses (Figure 13), suggests that the RHOCS pathway involving PknG, and RenU regulates cellular redox homeostasis through an NAD(H)-related mechanism. Interestingly, while RenU readily hydrolyzed NADH, the reduced form of nicotinamide adenine dinucleotide, it did not show significant catalytic activity towards the oxidized form, NAD⁺ (Figure 16).
Figure 16. RenU hydrolyses NADH preferentially over NAD$^+$ *in vitro*. Rate of RenU catalytic activity on NADH compared to its oxidative form NAD$^+$. Nonlinear least squares fit to the Michaelis-Menten equation is shown for NADH. Error bars represent standard deviation of triplicates.

### 3.2.3. PknG phosphorylates the L13 ribosomal subunit

To assess whether PknG interacts with RenU, their encoding genes were cloned for expression in *M. smegmatis* or *E. coli* as hexahistidine (6H) tagged proteins. Purified RenU.6H preparations were subjected to *in vitro* kinase assays using radioactive [$\gamma$-P$^{32}$]-ATP as the phosphate donor. Whereas the *M. smegmatis*-derived RenU.6H displayed a protein species phosphorylated by PknG, the *E. coli*-derived equivalent did not show phosphorylation (Figure 17). The MW of the phosphorylated protein species found in the *M. smegmatis* protein purification appeared ~5 kDa smaller than RenU.6H, as revealed by Coomassie Blue stained gels (Figure 17). This phosphorylated protein was identified by mass spectrometry as the 50S ribosomal protein L13 (or RplM, MW 16,119).
Figure 17. The L13 ribosomal subunit is associated with RenU

Representative in vitro phosphorylation of RenU.6H preparations purified from M. smegmatis (left) or E. coli (right) by purified PknG. PI, phosphatase inhibitors. Samples were separated by SDS-PAGE followed by blotting and autoradiography.

The M. smegmatis gene encoding L13 was cloned and expressed in E. coli as a hexahistidine tagged protein. The recombinant protein was purified and subjected to in vitro phosphorylation assays. These experiments showed that L13 was readily phosphorylated by PknG (Figure 18). The MW displayed by L13 on Coomassie Blue gels and autoradiographs was identical to the protein previously found to associate with RenU purified from M. smegmatis (Figure 18, lanes 5 and 8). Without PknG, L13 proteins from either M. smegmatis or Mtb showed no sign of phosphorylation in the presence of [γ-P32]-ATP (Figure 18, lanes 4), showing that the phosphorylation requires PknG. Furthermore, addition of the PknG specific inhibitor AX20017 inhibited the phosphorylation of L13 by PknG Figure 18, lane 6). Addition of a 6H-tag shifted the phosphorylated signal of L13 visualized on autoradiograph and Coomassie Blue stained gel (Figure 18, lane 7). These results confirmed the phosphorylation of L13 by the kinase activity of PknG.
Figure 18. The L13 ribosomal subunit is phosphorylated by PknG. 
*In vitro* phosphorylation of recombinant or native L13 protein associated with RenU by PknG kinase activity. The autoradiograph (top) and SDS-PAGE gel (bottom) are shown.

3.2.4. **Phosphorylation by PknG promotes the cytoplasmic association of L13 with RenU and its NADH hydrolytic activity**

To identify the specific amino acid residues that are phosphorylated by PknG, *Mtb* L13 protein purified from *E. coli* was subjected to a cold kinase assay catalyzed by PknG. L13 was then digested with trypsin and the derived peptides were analyzed by ISL-TOFF mass spectrometry (Taplin Biological Mass Spectrometry Facility, Harvard Medical School). This analysis suggested that one phosphorylated residue was present among the three
amino acids closely situated at positions 11–14 of the N-terminus of L13. Among these, T12 is absolutely conserved in all available bacterial L13 protein sequences, whereas T11 and S14 are exclusively conserved across the *Mycobacterium* genus. Mutant L13 proteins with inactivating mutations in these residues were made and the purified proteins were individually re-tested in *in vitro* phosphorylation assays. Whereas L13(T12A) and L13(S14A) mutants were readily phosphorylated (Figure 19, lanes 7 and 8), L13(T11A) completely failed to be phosphorylated by PknG (Figure 19, lane 6) similar to the triple mutant L13(3A) (Figure 19, lanes 5). These results indicate that the mycobacterial conserved T11 of L13 is uniquely phosphorylated by PknG.

**Figure 19. Phosphorylation of L13 and its mutants by PknG.**

In L13(3A), all three residues (T11, T12, and S14) were mutated to alanine. Inhibition was achieved by pre-incubation of PknG in 1 mM AX20017 (AX). Samples were separated by SDS-PAGE followed by blotting and autoradiography.

To better understand the role of the phosphorylation of L13 by PknG, we first analyzed if phosphorylation affects the formation of the L13-RenU complex in the mycobacterial cytoplasm. *M. smegmatis* strains representing different states of L13 phosphorylation were
first exposed to NADH to induce PknG expression. Cell lysates were prepared and ribosomes removed by ultracentrifugation. RenU.6H was then added to the non-ribosomal fractions. After incubation, RenU.6H was purified using Cobalt agarose beads and the copurification of L13 analyzed by Western analysis using a polyclonal anti-L13 antibody. Whereas RenU.6H was equally detected, L13 association with RenU.6H in the cytoplasm was dependent on its phosphorylation by PknG (Figure 20). This experiment suggests that phosphorylation of L13 at T11 by PknG promotes its association with RenU in the mycobacterial cytoplasm.

![Western blot analysis](image)

**Figure 20. Phosphorylation of L13 by PknG promotes formation of a RenU-phosphoL13 complex.**

Effect of PknG-catalyzed phosphorylation of L13 on its association with RenU in the cytoplasm. Expression of PknG in *M. smegmatis* strains was induced by NADH. Cells were disintegrated by French Press, followed by ultracentrifugation to remove ribosomes. RenU.6H was added to the non-ribosomal fraction, followed by pull-down using Cobalt-agarose beads. The presence of L13 in the pulled down materials was detected by Western analysis using anti-L13 antibody.

Because the function of RHOCs is involved with regulation of cellular NADH levels (Figure 15 and Figure 23), we examined if phosphorylated L13 affects the RenU-catalyzed
NADH hydrolysis (Figure 16). To study this question, we first established a fluorescence-based assay that allowed continuous monitoring of NADH hydrolysis by RenU. The assay was based on the different spectral characteristics of folded and unfolded conformations of NADH in aqueous solutions as previously reported [65]. NADH absorbs light at a wavelength of 260 nm through its adenine moiety and emits light at a wavelength of 460 nm through its nicotinamide moiety. The efficiency of the energy transfer responsible for this excitation/emission characteristic is decreased in the unfolded conformation or, in the case of this assay, upon the hydrolysis of NADH.

The hydrolysis of NADH by RenU in the presence or absence of L13(T11E), a mimic form of phosphorylated L13, was monitored. The initial rates were fit to the Michaelis-Menten equation (Figure 21) to determine $K_m$ and $V_{max}$ values (Table 3). Analysis of $K_m$ confirmed that there was no effect of L13(T11E) on RenU’s NADH-binding affinity (one-way ANOVA, effect of L13(T11E), $F(3,8)=2.06$, $p>0.18$) (Table 3).
Figure 21. Effect of L13(T11E), a phosphorylation-mimic form of L13, on \textit{in vitro} NADH hydrolytic activity of RenU

Initial rates from a continuous fluorescence excitation assay were fit by nonlinear least squares to the Michaelis-Menten equation to determine $K_m$ and $V_{max}$ values for RenU. Reaction was performed at 37°C. Error bars represent standard deviations of triplicates. The extent of the uncatalyzed reaction was \(~10\%\) of the RenU catalyzed reaction.

Table 3. Kinetic parameters of L13(T11E), a phosphorylation-mimic form of L13, on \textit{in vitro} NADH hydrolytic activity of RenU.

<table>
<thead>
<tr>
<th></th>
<th>$V_{max}$ [nM/s]</th>
<th>Effect on $V_{max}$</th>
<th>$K_m$ [µM]</th>
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<tr>
<td>Control</td>
<td>268.5±33.6</td>
<td>+20.60%</td>
<td>76.3±19.5</td>
</tr>
<tr>
<td>L13(T11E)</td>
<td>323.9±17.2</td>
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</table>

Furthermore, L13(T11E) did not have observable effects on NADH hydrolysis in the absence of RenU (Figure 21, blue vs green); and wild type L13 did not have observable effects on the NADH hydrolysis catalyzed by RenU (Figure 22A). Importantly, analysis of $V_{max}$ of the reactions performed at 37°C displayed a 20.6% increase in the rate of NADH
hydrolysis in the presence of L13(T11E) (one-way ANOVA, effect of L13 (T11E), F(3,8) = 35.80, p<0.05×10⁻³) (Figure 21, red vs black). An increase in V_{max} was also observed with temperature increments up to 42°C (Figure 22B). Together, these data suggest that the phosphorylation of L13 by PknG directly impacts not only the association of L13 with RenU in the mycobacterial cytoplasm, but also the NADH hydrolytic activity catalyzed by RenU.

Figure 22. Effect of L13 phosphorylation on in vitro NADH hydrolysis by RenU. A) Effect of wild type L13 on NADH hydrolysis by RenU. Initial rates from a continuous fluorescence excitation assay were fit by nonlinear least squares to the Michaelis-Menten equation. Error bars represent standard deviations of triplicates. B) Effect of L13(T11E) on the RenU-catalyzed NADH hydrolysis at different temperatures. Initial rates from a continuous fluorescence excitation assay were fit by nonlinear least squares to the Michaelis-Menten equation. Error bars represent standard deviations of triplicates.

3.2.5. RHOCS senses and regulates NADH cellular concentration
and is required for survival of M. tuberculosis in host macrophages

To elucidate if interruption of RHOCS activities affects cellular NADH levels, M. smegmatis mutants of the PknG-L13-RenU axis and the parental strain mc²155 were challenged with H₂O₂, followed by extraction and analysis of NADH, NAD⁺, and FAD
concentrations. Whereas interruption of RHOCs did not affect NAD$^+$ level, it resulted in dramatic accumulations of NADH and FAD by H$_2$O$_2$ (Figure 23).

**Figure 23. Interruption of RHOCs causes NADH and FAD accumulation**

Quantitation of cellular NADH (top), NAD$^+$ (middle), and FAD (bottom) levels following oxidative stress induced by H$_2$O$_2$. *M. smegmatis* cells were exposed to 1 mM H$_2$O$_2$ for 1 hour. Bars show means with standard deviations from 3–6 biological repeats. *, p < 0.0001; ns, not significant relative to wild type *M. smegmatis.*

To analyze if the intracellular survival of *Mtb* strains correlated with their lysosomal delivery levels, trafficking of internalized *Mtb* strains was analyzed by microscopy. As shown previously [54], absence of *pknG* resulted in increased lysosomal delivery (Figure 24B). The *MtbΔpknG* mutant was largely localized within acidic milieu, whereas wild type *Mtb* displayed a low level of localization within lysosomes (Figure 24B). *In trans* expression of *pknG* restored wild type level of lysosomal delivery to *MtbΔpknG* (Figure 24B). In addition, *MtbΔrenU* and *Mtb.L13(T11A)* mutants exhibited lysosomal delivery levels comparable to that of *MtbΔpknG* (Figure 24B) while the corresponding complemented strains, *MtbΔrenU/renU* and *L13(T11A)/T11E*, behaved like wild type in lysosomal delivery. In an agreement with the survival (Figure 24A), RenU$^\text{DEAD}$, the
catalytically inactive mutant, failed to rescue $Mtb\Delta renU$ and the $L13(T11A)/(T11E)$ strain showed resistance to AX20017 (Figure 24A). These results suggest that the function of RHOCS in $Mtb$ lysosomal delivery is correlated, either as a cause or a consequence, to the survival of the bacillus in the macrophage.

**Figure 24. Intracellular trafficking and survival of $M. tuberculosis$ RHOCS mutants.**

A) Intracellular survival of $Mtb$ strains. Macrophages were infected with $Mtb$ strains for 3 hours, followed by 0 or 72-hour chase. CFUs were counted after 4–5 weeks of growth at 37°C. Bars represent percentages of CFUs remaining at 72-hour compared to 0-hour time point. Error bars represent standard deviations from 3-6 repeats. *, p < 0.001; ns, not significant relative to $Mtb$ H37Rv; #ns, not significant between the two indicated groups. Order of strains is as in panel B. B) Quantitative analysis of lysosomal delivery following phagocytosis of $Mtb$ strains by macrophages. Macrophages were infected with FLUOS-stained $Mtb$ strains for 1 hour, followed by 16-hour chase. Infected macrophages (see C below) were used for quantitation. Biological triplicates of 50 events were counted for each $Mtb$ strain. Error bars represent standard deviations. *, p < 0.0001; ns, not significant relative to $Mtb$ H37Rv; ** ns, not significant between the two indicated groups.
This work has revealed a novel signaling mechanism that is used by mycobacteria to regulate cellular redox homeostasis (Figure 25). We propose that this system, RHOCS, is capable of sensing the key redox regulator NADH, and regulating its cellular level through direct degradation. RHOCS is composed of at least three components: a eukaryotic-type protein kinase, PknG, a ribosomal protein, L13, and a Nudix hydrolase, RenU. RHOCS is responsive to cellular NADH levels through up-regulated expression of PknG, which phosphorylates L13 at a unique site, T11, and promotes its cytoplasmic association with RenU. At least two possible mechanisms may lead to this increased L13-RenU association: (i) phosphorylation of L13 by PknG prevents the association of L13 with the ribosome, or (ii) the phosphorylation causes releases of L13 from the ribosome, similar to the effect of L13a phosphorylation by ZIPK observed in human macrophages [66]. Once associated with RenU in the cytoplasm, phosphorylated L13 accelerates the Nudix hydrolase activity of RenU that directly degrades NADH, thus lowering its cellular level. This paradigm of redox control is novel and has not been observed before in bacteria.

The fact that both Mtb and its host macrophage use L13 phosphorylation as a common method to convey cellular stress responses is fascinating and warrants further investigation. Our work also supports the recent “depot hypothesis”, which proposes that macromolecular complexes such as the ribosome function as “reservoirs” for regulatory proteins that perform non-canonical functions [67, 68].

The mechanism of action proposed for RHOCS (Figure 25) fits nicely with some of the previous observations. Work by other groups suggests that PknG derepresses the TCA cycle through its phosphorylation of the cycle inhibitors GarA or OdhI [69, 70]. Thus, activity of PknG in the TCA cycle is expected to increase production of NADH, which is
then fed into the oxidative phosphorylation pathway that produces reactive oxygen species and free radicals. In addition, NADH is an effective inhibitor of α-ketoglutarate dehydrogenase, the key generator of NADH and oxidative stress [71], and a target of GarA [69, 70]. Therefore, the role of PknG in the RHOCS pathway may provide mycobacteria with a supportive mechanism that prevents cell death from redox disturbance caused by increased TCA activity. Interestingly, free radicals produced from NADH by the TCA cycle were recently suggested to mediate bacterial cell death triggered by bactericidal antibiotics [72]. Accordingly, the function of RHOCS in NADH control may also help to explain the recent observation that absence of PknG leads to enhanced antibiotic susceptibility in mycobacteria [55, 73]. These results, all together, indicated that the kinase activity of PknG, the Nudix hydrolase activity of RenU, as well as the phosphorylation of L13, each is required for the survival of Mtb in host macrophages.
PknG was previously shown to derepress the TCA cycle through its phosphorylation of GarA, an inhibitor of α-ketoglutarate decarboxylase and glutamate dehydrogenase. Increased TCA cycle activities, hypoxia, or impaired oxidative phosphorylation (OXPHOS), lead to elevated NADH levels. To protect mycobacterial cells against the change in redox status, PknG expression is up-regulated, leading to the signaling cascade including L13 and RenU, which degrades NADH and FAD and restores their optimal level. AMP, adenosine monophosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NMNH, nicotinamide mononucleotide.

3.3. Structural studies

Crystallization trials of wild type RenU, RenU.6H, 6H.RenU, and the surface entropy reduction EQ84AA mutant failed to produce atomic-resolution-diffraction quality crystals. The RenU E(74, 77, 78)A mutant readily crystallized as thin plate clusters in hanging drop vapor diffusion trials at 20 °C. The drop contained 1 μl of a 500 μl reservoir (1-1.4 M NaAc, 0.1 M BTP pH 7.5) and 1 μl of 15 mg/ml of enzyme in stock buffer (50 mM Tris-HCl pH 8.6). The thin plates were crushed with a needle after 2-weeks of growth, vortexed in stock buffer, and added to the protein stock in a 1:2000 ratio. Hanging drop vapor diffusion trials as described above, but using the seeded protein stock, resulted in a 2.25 Å resolution diffraction dataset with 91.8 % completeness (Table 4). Crystallographic data
was collected with the use of cryoprotectant at beamline 5.0.2 at the Advanced Light Source at Lawrence Berkeley National Laboratory.

Table 4. RenU crystallographic data collection statistics.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Apo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
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</tr>
<tr>
<td>Cell dimensions(Å)</td>
<td></td>
</tr>
<tr>
<td>$a$, $b$, $c$ (Å)</td>
<td>34.7, 118.7, 36.3</td>
</tr>
<tr>
<td>$\alpha$, $\beta$, $\gamma$ (°)</td>
<td>90.0, 92.9, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
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</tr>
<tr>
<td>$R_{symm}$ (%)</td>
<td>(2.33-2.25)</td>
</tr>
<tr>
<td>$I/\sigma$</td>
<td>33.73 (2.96)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>91.8 (54.2)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.2 (2.0)</td>
</tr>
</tbody>
</table>

Attempts to solve the phase problem using heavy atom replacement (lanthanides) and determine the RenU atomic structure are currently underway.
CHAPTER 4

Nud1p, a Coenzyme A hydrolase in *T. thermophila*
Regulation of eukaryotic gene expression is mediated in part through variations in the structure of chromatin, a highly organized physiological state of genetic material that is formed by the interaction of DNA and proteins, mostly histones. The fundamental subunit of chromatin is the nucleosome, consisting of 146 bp of DNA wrapped around an octameric histone core that contains a tetramer of the histones H3 and H4, and two dimers of the histones H2A and H2B. Chromatin in the transcriptionally “off” state is characterized by densely compacted nucleosomes and is known as heterochromatin, while the transcriptionally “on” state is a generally less compacted form known as euchromatin. Covalent post-translational modifications (PTM) on histones, such as methylation and acetylation help control the functional shift between silent and active state. In this way, histone PTMs have been implicated as regulators of epigenetic phenomena, although the mechanistic details are poorly understood [74]. One way to gain insight into epigenetic regulation is to characterize the protein complexes that “write”, “erase”, and “interpret” histone PTMs.

The protein complexes that “write” histone PTMs, as well as the histone sequences themselves, are highly conserved throughout evolution. One such complex of proteins, called the Polycomb complex, is known to be conserved in metazoans, and functions in gene silencing. Polycomb mediated H3K27me3, as in developmental Hox gene silencing and X-chromosome inactivation [74], is thought to recruit proteins that bind to H3K27me3 which reorganize chromatin structure and shut down transcription. While studying the Ezl1p Polycomb complex in *Tetrahymena thermophila*, Taverna et al discovered a novel protein member of the complex (Figure 26). The function of this protein, encoded by the gene *NUDI* and annotated as Nud1p, could reveal additional insight into the Polycomb
group mediated gene silencing. Sequence analysis of the gene encoding Nud1p revealed two uncharacterized domains flanking a Nudix fold. Sequence elements neighboring the Nudix signature motif resemble those of a Nudix Coenzyme A hydrolases [75, 76], suggesting that CoA may be the endogenous Nud1p substrate.

Figure 26. Nud1p is a novel member of the Ezl1p complex
A) Figure courtesy of S. Taverna and R. Papazyan (Johns Hopkins University, Baltimore, MD) and Yifan Liu (University of Michigan, Ann Arbor, MI). Co-immunoprecipitation of Ezl1p-FLAG-HA from T. thermophila followed by mass spectrometry shows the components in the Ezl1p complex. B) Representation of the Ezl1p complex showing Nud1p, a CoA Nudix hydrolase.

All genomes sequenced to date reveal enzymes that utilize Coenzyme A (CoA) or its conjugate form, in which the thiol group of CoA forms a bond with an ester. This way, CoA is able to transport acetate and fatty acids for use in many cellular processes throughout the cell: in the mitochondrion, CoA shuttles an acyl group from glycolysis or β-oxidation as fuel into the tricarboxylic acid cycle; in the cytosol, CoA mediates the transfer of phosphopantetheine to the acyl carrier protein; and in the nucleus, it mediates the transfer of acetate to histones. In mice, Nudt7[77] and Nudt19[76] can regulate CoA levels by degrading it into AMP and phosphopantetheine. Similar Nudix hydrolyzes exist
in other organisms and likely exert CoA regulatory functions. In *Deinococcus radiodurans*, an extremophile resistant to high levels of gamma, UV, and oxidative stress, *Dr1184* is responsible for the expression of a Nudix CoAase that degrades oxidized forms of CoA [75]. In bacteria, degradation of oxidized CoA is particularly important when the reducing environment of the cytosol is challenged by oxidative stress, oxidizing the otherwise reduced forms of CoA.

4.1. **In vitro studies**

To investigate whether Nud1p hydrolyzed CoA, the wild type enzyme was purified to homogeneity and a variety of adenosine diphosphate derivatives were assayed as described in section A1.1. Nud1p’s solution oligomerization state (monomer) and purity were assessed by gel filtration chromatography (Figure 27).

![Figure 27. Gel filtration chromatography indicates Nud1p is a monomer in solution. Gel filtration chromatography on a Sephacryl 16/60 S-200 column. Shown are elution profiles of Nud1p (solid, ~46 kDa) and two chromatography standards: carbonic anhydrase (dotted, 29 kDa) and albumin (dashed, 66 kDa).]
Nud1p and two other Coenzyme A hydrolases, Nudt7[77] and Dr1184[75], exhibited negligible rates of relative substrate hydrolysis for NADP, NADH, and NAD (Figure 28). Importantly, Nud1p had a relative hydrolysis approximately four times higher than Dr1184 (Figure 28, grey vs green), demonstrating that Nud1p is a CoA hydrolase. Nud1p displayed significantly lower relative hydrolase activity toward NTPs including ATP, GTP, UTP or other NDPXs such as AP4A, GDPM, and ADPR.

![Figure 28](image.png)

**Figure 28. Nud1p relative Nudix hydrolase activity.**
Nud1p (gray) and two other Nudix Coenzyme A hydrolases, Nudt7 (red) and Dr1184 (green), were assayed for relative hydrolytic activity against a panel of adenosine diphosphate derivatives.

Due to its notable role in many cellular processes as an acyl carrier, we explored whether CoA thioesters were hydrolyzed by Nud1p. To our surprise, acetyl-CoA and acetoacetyl-CoA were both hydrolyzed at significantly lower levels than the reduced form of CoA (Figure 29A). Analysis of the initial rates of hydrolysis confirmed Nud1p’s substrate preference for CoA over the thioester forms of this cofactor (Figure 29B). Substitution of
the glutamate residues $E_{16}^N$, $E_{19}^N$, and $E_{20}^N$ (E164, E167, and E168) in the signature motif on helix αM for alanine E(164, 167, and 168)A, completely abolished the enzymatic activity of Nud1p (Figure 29A, purple). The point mutant E164 retained a significant relative hydrolysis (~90%) when compared to wild type enzyme (Figure 29A, red).

**Figure 29. Nud1p wild type and mutant relative Nudix hydrolase activity.**
A) Nud1p (grey) and its mutants, E164A (red) and E(164,167,168)A (blue), were assayed against a panel of CoA and its acetyl and acetoacetyl derivatives (AcCoA and AcAcCoA, respectively). B) Nud1p initial rates of hydrolysis for CoA (—), AcCoA (□), and AcAcCoA (●).

### 4.2. *In vivo studies*

*I. thermophila*, a ciliate protozoan, presents an elegant model to study epigenetic regulation due to its nuclear dimorphism and biphasic life cycle. *T. thermophila* contains two distinct nuclei with different functions: a germline micronucleus (MIC) responsible for gene expression during sexual replication; and a somatic macronucleus (MAC) responsible for gene expression during vegetative growth. It is this compartmentalization of genes characteristic of the vegetative or sexual phases that reveals information about a particular gene’s function. In the case of the *EZLI* gene, its function can be elucidated by observing the change in methylation state of chromatin during epigenetic silencing of anlagen, the newly developed macronuclei. *EZLI* encodes the
methyl transferase Ezl1p. This enzyme forms the core of the Ezl1p complex and “writes” the “off” PTM (trimethylation or me3) on histones characteristic of the transcriptionally silent heterochromatin (H3K27me3 and H3K9me3).

When any one of the canonical members of the Ezl1p complex is deleted, *T. thermophila* loses the ability to trimethylate H3K27 and H3K9 during anlagen formation, indicating that heterochromatin formation and its consequent gene silencing are impaired. Interestingly, deletion of *NUD1* results in the same phenotype observed in ∆EZL1, thus confirming Nud1p’s presence in the Ezl1p complex.

**Figure 30.** Nud1p and other members of the *Tetrahymena* Ezl1p complex are required for histone methylation and heterochromatin formation.

Figure courtesy of S. Taverna and R. Papazyan (Johns Hopkins University, Baltimore, MD) and Yifan Liu (University of Michigan, Ann Arbor, MI). Immunofluorescence images of *Tetrahymena thermophila* during early anlagen. The apoptotically degrading old macronuclei (white arrow), the micronuclei (white arrowheads), and the new macronuclei (green arrows) are shown. The loss of green signal in the new macronuclei shows that cells lose the ability to methylate H3K27 (top) and H3K9 (bottom), both indicators of heterochromatin formation, when any of the Ezl1p complex members is knocked out. The DNA is stained blue using DAPI. Anti-H3K27me3 and anti-H3K9me3 antibodies are green.

It remains to be determined if the observed phenotype is a result of: (i) loss of the Nudix CoA hydrolase activity; (ii) unsuccessful complex assembly because of the lack of an
integral protein member; and/or (iii) failure of the complex to localize to anlagen. Point (i) can be answered with a Nud1p E(164, 167, 168) T. thermophila strain and establish whether CoA concentrations are as relevant as histone deacetylases, methyl transferases, and acety transferases in regulating histone acetylation as shown by Siudeja [78]. A co-immunoprecipitation experiment as in Figure 26 from a ΔNud1p mutant could answer point (ii) by showing if the other members of the complex are present after immunoprecipitation of Ezl1p. Lastly, point (iii) can be answered with transmission electron microscopy immunohistochemistry as in Figure 3 using an anti-FLAG antibody to identify Ezl1p localization during anlagen formation in ΔNUD1 and Nud1p E(164, 167, 168)A strains.

4.3. Structural studies

Exhaustive Nud1p crystallization trials of wild type, E164A, and ΔN-terminus mutants with a variety of substrates (Figure 28 and Figure 29) in the absence (EDTA) or presence of metal cofactors (Ca²⁺, Mn²⁺, and Mg²⁺) failed to produce atomic-resolution-diffraction quality crystals. Reducing agents (TCEP or DTT) at 2-5 mM concentrations in the vapor diffusion reservoir were required to prevent formation of protein precipitant at the air-liquid interface. The catalytically inactive E(164, 167, 168)A Nud1p mutant formed thin crystal plates after 3 weeks of incubation at 20 °C over 500 μl of reservoir (17-22% PEG 1500, 0.1 M HEPES, pH 7.5, 5% glycerol, 4 mM TCEP). The crystals diffracted to ~2.8 Å, but had low durability with deformation and crosslinking to neighboring precipitant in 1-2 weeks after crystal formation. Furthermore, the diffraction patterns were anisotropic and characteristic of high mosaicism, making determination of the space group and cell parameters untenable (Figure 31).
Figure 31. Nud1p crystal plates diffract at atomic resolution. A) Hanging drop vapor diffusion crystal plate of Nud1p E(164, 167, 168)A. B) Nud1p E(164, 167, 168)A Cu-Kα rotating anode X-ray diffraction on an R-axis IV detector.

To increase Nud1p E(164, 167, 168)A crystal and diffraction quality, we developed and conducted organic inhibitor assay (Appendix D1) to find compounds which could increase the conformational homogeneity of Nud1p by binding to the active site. We identified 8 compounds from the NIH Clinical Collection Screen (Evotec, Inc) with disassociation constants (Table 5) comparable to the $K_m$ observed in other Nudix-substrate pairs (Table 2 and Table 3). Crystal optimization of the Nud1p E(164, 167, 168)A mutants using these inhibitors is currently underway.

### Table 5. Nud1p NIH Clinical Collection inhibitor dissociation constants

<table>
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<tr>
<th>Compound</th>
<th>SRP0120P</th>
<th>SRP0125r</th>
<th>SRP00530i</th>
<th>SRP00675i</th>
<th>SRP01172i</th>
<th>SRP01061a</th>
<th>SRP01568i</th>
<th>SRP01010v</th>
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</thead>
<tbody>
<tr>
<td>$K_d$ (µM)</td>
<td>68</td>
<td>101</td>
<td>98</td>
<td>85</td>
<td>99</td>
<td>95</td>
<td>61</td>
<td>109</td>
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</table>
CHAPTER 5

Sequence based characterization
Comparison of the sequences, atomic structures, and substrates of Bd-NDPSase, Ec-GDPMase, and Ec-ADPRase allowed us to identify sequence elements to differentiate and categorize these enzymes into families. Importantly, we recognized that Ec-GDPMase is not just a GDPM hydrolase, but, like Bd-NDPSase, hydrolyzes a variety of nucleoside diphosphate sugars. Thus, these two enzymes form part of the novel NDPSase Nudix family. This family is characterized by a domain swapped dimer with a hydrophobic residue in loop L-2 and an aspartate-lysine bridge at the N-terminus of αS2 (Figure 4 and Figure 32). Despite the lack of atomic resolution structural information about Nud1p and the presence of two uncharacterized flanking domains, we identified a serine/threonine-proline pair located in loop LS which is a likely predictor of CoA hydrolase Nudix hydrolases (Figure 32).

**Figure 32. Sequence elements characteristic of the NDPS, ADRP, and CoA Nudix hydrolase families.**

Sequence elements are colored as in Figure 1. Shown are residues in the N-terminal domain (green), residues in loop LS (green), conserved residues in the signature motif (cyan), and residues in helix αS2 (grey).

Herein, we have identified Nudix structural elements that are strong determinants of substrate recognition. Based on these findings, we proposed that, when lacking structural information, loop LS can be used to guide sequence based characterization of Nudix families and their members. This loop is readily identified from primary sequence as it is ~15 residues downstream from the signature motif and links β5 and β6, both of which are often identified with secondary structure prediction. Likewise, helices αS1 and αS1 and
their adjoining loop could be identified through secondary structure prediction to improve sequence based characterization.
Appendix A  –  Frequently used methods

A1.  Enzyme Assays

A1.1. Substrate determination assays

The enzymatic activity and substrate specificity of the Nudix enzymes were investigated using a coupled enzyme colorimetric assay [79]. For nucleoside diphosphate derivatives (NDPX), these calf intestinal alkaline phosphatase (CIP) insensitive substrates were first converted to CIP-sensitive products by Nudix hydrolysis, followed by the release of phosphate by CIP. For nucleoside triphosphates (NTP), the release of phosphate was catalyzed by inorganic pyrophosphatase (PP\_i\_ase). In both cases, phosphate was measured by the method of Ames and Dubin [80].

The standard incubation mixture contained, in 50 µl, 50 mM Tris pH 8.4, 5 mM MgCl\_2, and 150-250 nM purified Nudix enzyme. The mixture also contained 2 mM of a NDPX and 20 U/ml CIP, or 2 mM of a NTP and 20 U/ml PP\_i\_ase. After 15 minutes at 37°C, the reactions were terminated by the addition of 30 µl of 100 mM Na-EDTA. The stopped reactions were kept on ice for 5 minutes, followed by the addition of 700 µl of Ames solution (6 parts 0.42% ammonium molybdate in 1 N H\_2SO\_4 and 1 part 10% succinic acid) [80]. The mixtures were then incubated for 20 minutes at 42°C. The relative amount of phosphomolybdate was determined by absorbance at 820 nm.
A1.2. Kinetics assays

The phosphate release assay (section A1.1) was used to determine the kinetics parameters of Nudix substrate hydrolysis. 50 μl of a 350 μl reaction solution consisting of nucleoside diphosphate derivative at varying concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1 mM), 50 mM Tris-HCl pH 8.6, 1 mM EDTA buffer, 5 mM MgCl2, 2.5 mM enzyme, and calf intestinal phosphatase (New England Biolabs, Inc.) were taken every 2 minutes. The reaction for each time point was quenched with 30 μl of 100 mM EDTA and placed on ice. 220 μl of ddH2O was added to each time point, followed by the addition of 700 μl of Ames solution. These were developed at 42 °C for 20 minutes. The concentration of the phosphate liberated was measured at A820. A standard curve was used to calculate the amount of hydrolyzed substrate. Initial rates of substrate hydrolysis were fit by nonlinear least squares to the Michaelis-Menten equation to determine kcat and Km.

A2. Protein Expression and Purification

A2.1. Bd-NDPSase protein expression and purification

BL21 (DE3) cells were transformed with a pET24a (Novagen, Madison WI) plasmid containing the *Bdellovibrio bacteriovorus* Bd3179 gene. Cells were grown at 37 °C from a single colony inoculum in LB media supplemented with Kanamycin. Bd-NDPSase expression was induced at an OD600 of 0.7 with 100 μM IPTG. Cells were grown for three to five hours, centrifuged, pelleted, and frozen at -80 °C.

The cell pellet was thawed, resuspended in 80 ml TE buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA), and lysed with a microfluidizer 2x at 75 psi. The cell lysate was clarified by centrifugation for 30 minutes at 11,500 rpm using TE buffer at 0, 30, and 60% ammonium sulfate concentrations. The pellet from the last clarification step was resuspended in TE
buffer at a 60% ammonium sulfate concentration, filtered with a Corning PES 0.20 µm filter, and loaded on a hydrophobic interaction column (Phenyl FF HiPrep 16/10 hydrophobic interaction column, GE Healthcare) equilibrated in resuspension buffer. Bound protein was eluted with a TE buffer gradient. Fractions containing protein were dialyzed at 4 °C 2x against 2 L of 50 mM Tris-HCl pH 8.6, 1 mM EDTA buffer overnight in 3500 Da dialysis tubing (SnakeSkin, Thermo Scientific), and loaded onto an anion exchange column (Resource Q, GE Healthcare). The protein was loaded with 50 mM Tris-HCL pH 8.6, 1 mM EDTA (Buffer A) and eluted with a 50 mM Tris-HCl pH 8.5, 1 mM EDTA and 1 M NaCl (Buffer B) gradient. Combined eluted fractions were concentrated and the purity of the sample (>90%) was assessed by SDS-PAGE.

**A2.2. RenU protein expression and purification**

For expression and purification of *M. smegmatis* RenU, RenU E(164)A, or RenU E(164,167,168)A in *E. coli*, BL21 cells transformed with pET11c or pET15b were grown at 37°C until OD$_{600}$ reached 0.5, followed by induction with 0.5 mM IPTG at 30°C, 250 r.p.m. for 3 hours. Cell lysates were prepared by sonication in lysis buffer (50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole) containing protease inhibitor cocktail (15 cycles of 10 seconds on ice with chilling intervals). After centrifugation, the soluble fraction was loaded onto a nickel metal affinity spin column (Qiagen), which had been pre-equilibrated with the same buffer. The column was washed 6 times with 2 column volumes of wash buffer (50 mM sodium phosphate, 300 mM NaCl, and 20 mM imidazole) and bound protein was eluted with 4 column volumes of elution buffer (50 mM sodium phosphate, 300 mM NaCl, and 500 mM imidazole). Eluted fractions were pooled and
stored at -80°C with 10% glycerol. Renu and related mutants protein purification steps are detailed in Table 6.
### Table 6. RenU purification protocol

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
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<td><strong>Lysis buffer (100 ml):</strong></td>
<td>Take 100 ml of buffer A in step 2 Add 2 roche pills or 200 μl of 50 mM PMSF</td>
</tr>
<tr>
<td><strong>Ni-NTA/Hexahistidine binding/wash Buffer A (600 ml):</strong></td>
<td>H₂O (472 ml) 50 mM Tris pH 8.4 (30 ml of 1 M), 3 mM MgCl₂ (1.8 ml of 1 M) 500 mM NaCl (60 ml of 5 M), 20 mM Imidazole (6 ml of 2M) 5% glycerol (30 ml)</td>
</tr>
<tr>
<td><strong>Ni-NTA/Hexahistidine elution Buffer B (200 ml):</strong></td>
<td>H₂O (130 ml) 50 mM Tris pH 8.4 (10 ml of 1 M), 3 mM MgCl₂ (600 μl of 1 M) 500 mM NaCl (20 ml of 5 M), 300 mM Imidazole (30 ml of 2M) 5% glycerol (10 ml)</td>
</tr>
<tr>
<td><strong>Dialysis into Thrombin cleavage buffer pH 8 (2L):</strong></td>
<td>H₂O (to 2L) 50 mM Tris pH 8 (100 ml of 1 M) 150 mM NaCl (60 ml of 5 M) 2.5 mM CaCl₂ (5 ml of 1 M) 5 mM BME (700 μl of 14.2 M)</td>
</tr>
<tr>
<td><strong>Dialysis (Mono Q) buffer (2 L):</strong></td>
<td>H₂O (1594 ml) 50 mM Tris pH 8.4 (100 ml of 1 M) 3 mM MgCl₂ (6 ml of 1 M) 100 mM Glycine pH 8.4 (200 ml of 1M) 5% glycerol (100 ml)</td>
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<tr>
<td><strong>ResourceQ binning/wash Buffer A (400 ml):</strong></td>
<td>Filter dialysis buffer, use as A</td>
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<tr>
<td><strong>Resource Q elution Buffer B (200 ml):</strong></td>
<td>H₂O (120 ml) 50 mM Tris pH 8.4 (10 ml of 1 M) 3 mM MgCl₂ (600 μl of 1 M) 1 M NaCl (40 ml of 5 M) 100 mM Glycine pH 8.4 (20 ml of 1M) 5% glycerol (10 ml)</td>
</tr>
<tr>
<td><strong>SEC Superose 12 Buffer A (300 ml):</strong></td>
<td>H₂O (237 ml) 50 mM Tris pH 8 (15 ml of 1 M) 200 mM NaCl (12 ml of 5 M) 10 mM BME (208 μl of 14.4 M) 100 mM Glycine pH 8.4 (30 ml of 1M) 1 mM EDTA (600 μl of 0.5 M)</td>
</tr>
</tbody>
</table>

**Add the buffer to the cold pellet to thaw**  
**Dounce homogenize and microfluidize**  
**Clarify at 15K RPM for 40 mins**  
**Equilibrate column with 4 CV of buffer A**  
**Load soluble lysate and allow to elute**  
**Elute with an A->B 20CV gradient**  
**Protein should elute before 60%B (200 mM I)**  
**Dialysis the same day in 1L for 2 hours twice**  
**Used 100 U/100 μl stock at 0.5 μl Thrombin per 1 ml of prep. 1 ml reactions in Eppendorf tubes. 15-30 minutes gave full cleavage of tag. Extra time gives nonspecific terminal cleavage.**  
**Dialysis the second day at 4 °C**  
**After second dialysis, filter the buffer for use in MonoQ (buffer A)**  
**Equilibrate column, load sample, and wash/hold as necessary**  
**Elute with a 20 CV gradient to 500 mM NaCl**  
**Clean/final elute with a step to 1 M NaCl**  
**Run gel and pool fractions**  
**Concentrate sample to 500 μl if possible to run SEC**  
**Equilibrate superpose 12**  
**Load sample and elute with 1.5 CV**  
**Concentrate protein to 8 mg/ml**
A2.3. L13(T11E) protein expression and purification

For expression and purification of *M. smegmatis* 6H.L13(T11E), *E. coli* BL21 cells were transformed with pVN931 and grown at 37°C, 220 r.p.m. until OD\(_{600}\) reached 2. This culture served as a seed to inoculate (1/100) LB medium supplemented with ampicillin. At an OD\(_{600}\) of 0.6, the inoculated cultures were treated with 0.5 mM IPTG to induce protein expression for 3 hours at 37°C, 220 r.p.m. Cell lysate was prepared in lysis buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, 5% glycerol, pH 8.4) containing protease inhibitor cocktail (Roche) by microfluidization. After centrifugation, the soluble fraction was loaded onto a nickel affinity column (Qiagen) pre-equilibrated with the same buffer. The column was washed 10 times with 2 column volumes of wash buffer (50 mM Tris, 500 mM NaCl, 50 mM imidazole, 5% glycerol, pH 8.4) and bound protein was eluted with 10 column volumes of elution buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole, 5% glycerol, pH 8.4). The eluted protein was dialyzed into MonoS binding buffer (50 mM Tris, 40 mM NaCl, 5% glycerol, pH 8.4) and loaded onto a MonoS column pre-equilibrated with the same buffer. Protein was eluted with a 20 column volume gradient to an equivalent buffer containing 500 mM NaCl. The eluted protein was pooled and concentrated using 3 kDa molecular weight cut-off spin concentrators (Millipore) for size exclusion chromatography on a Superose 12 column pre-equilibrated in running buffer (50 mM Tris, 300 mM NaCl, 5% glycerol, pH 8.4). Protein was pooled and concentrated as above to 0.61 mg/ml and stored at -80°C. L13 and related mutants protein purification steps are detailed in Table 7.
<table>
<thead>
<tr>
<th>Table 7. L13 purification protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysis buffer (100 ml):</strong></td>
</tr>
<tr>
<td>Take 100 ml of buffer A in step 2</td>
</tr>
<tr>
<td>Add 2 roche pills or 200 μl of 50 mM PMSF</td>
</tr>
<tr>
<td><strong>Ni-NTA/Hexahistidine binding/wash Buffer A (400 ml):</strong></td>
</tr>
<tr>
<td>H₂O (335 ml)</td>
</tr>
<tr>
<td>50 mM Tris pH 8.4 (20 ml of 1 M)</td>
</tr>
<tr>
<td>500 mM NaCl (40 ml of 5 M)</td>
</tr>
<tr>
<td>20 mM Imidazole (4 ml of 2M)</td>
</tr>
<tr>
<td><strong>Ni-NTA/Hexahistidine elution Buffer B (100 ml):</strong></td>
</tr>
<tr>
<td>H₂O (75 ml)</td>
</tr>
<tr>
<td>50 mM Tris pH 8.4 (5 ml of 1 M)</td>
</tr>
<tr>
<td>500 mM NaCl (10 ml of 5 M)</td>
</tr>
<tr>
<td>200 mM Imidazole (10 ml of 2M)</td>
</tr>
<tr>
<td><strong>Dialysis (Mono Q) buffer (2 L):</strong></td>
</tr>
<tr>
<td>H₂O (1883 ml)</td>
</tr>
<tr>
<td>50 mM Tris pH 8.4 (100 ml of 1 M)</td>
</tr>
<tr>
<td>40 mM NaCl (16 ml of 5 M)</td>
</tr>
<tr>
<td><strong>MonoQ bining/wash Buffer A (400 ml):</strong></td>
</tr>
<tr>
<td>Filtered dialysis buffer</td>
</tr>
<tr>
<td><strong>MonoQ elution Buffer B (200 ml):</strong></td>
</tr>
<tr>
<td>H₂O (150 ml)</td>
</tr>
<tr>
<td>50 mM Tris pH 8.4 (10 ml of 1 M)</td>
</tr>
<tr>
<td>1 M NaCl (40 ml of 5 M)</td>
</tr>
<tr>
<td><strong>SEC Superose 12 buffer A (300 ml):</strong></td>
</tr>
<tr>
<td>H₂O (267 ml)</td>
</tr>
<tr>
<td>50 mM Tris pH 8.4 (15 ml of 1 M)</td>
</tr>
<tr>
<td>300 mM NaCl (18 ml of 5 M)</td>
</tr>
</tbody>
</table>
A2.4. Nud1p protein expression and purification

Nud1p and related mutants were expressed in *E. coli* using a pGEX vector with a GST tag.

Nud1p yield was optimal when Nud1p expression was induced with 200 μM of IPTG at an optical absorbance of 0.8 OD<sub>600</sub> for 3 hours at 37 °C. Protein purification steps are detailed in Table 8.

### Table 8. Nud1p purification protocol

<table>
<thead>
<tr>
<th>Step</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysis buffer (100 ml):</strong></td>
<td>Take 100 ml of buffer A in step 2 Add 2 roche pills or 200 μl of 50 mM PMSF</td>
</tr>
<tr>
<td><strong>GST binding/wash Buffer A (500 ml):</strong></td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (425 ml) 50 mM PBS pH 7.4 (50 ml of 10X) 5% Glycerol (25 ml) 10 mM BME (350 μl of 14.3 M)</td>
</tr>
<tr>
<td><strong>GST Elution Buffer B (100 ml):</strong></td>
<td>Take 95 ml of Cleavage Buffer Add 20 mM Reduced Gluthathione (615 mg of 307.21 FW) Adjust pH to 8.5 (230 μl of 10M NaOH)</td>
</tr>
<tr>
<td><strong>Dialysis GST tag Cleavage Buffer (1L):</strong></td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (919) 50 mM Tris-HCL pH 8-8.5 (50 ml of 1M) 10 mM BME (700 μl of 14.3 M) 150 mM NaCl (30 ml of 5M) 10 μM EDTA (20 μl of .5 M)</td>
</tr>
<tr>
<td><strong>Dialysis (ResourceQ) buffer (2 L):</strong></td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (1888.6 ml) 40 mM NaCl (16 ml of 5 M) 50 mM Tris-HCl pH 8 (100 ml of 1 M), 10 mM BME (1.4 mL of 14.3 M)</td>
</tr>
<tr>
<td><strong>ResourceQ binig/wash Buffer A (400 ml):</strong></td>
<td>Filtered dialysis buffer Equilibrate column, load sample, and wash/hold as necessary. CV is 6 ml.</td>
</tr>
<tr>
<td><strong>ResourceQ elution Buffer B (200 ml):</strong></td>
<td>40/8 mM Tris/BME pH 8 (162 ml of buffer A) 1 M NaCl (38 ml of 5 M NaCl) Elute with a 20 CV gradient to 500 mM NaCl Clean/final elute with a step to 1 M NaCl Elutes at ~90 mS Concentrate sample to 500 μl if possible to run SEC (2 runs of 250-300 μl)</td>
</tr>
<tr>
<td><strong>SEC Superose 12 buffer A (300 ml):</strong></td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O (267 ml) 50 mM Tris pH 8 (15 ml of 1 M) 200 mM NaCl (12 ml of 5 M) 10 mM BME (208 μl of 14.4 M) 1 mM EDTA (600 μl of 0.5 M) Equilibrate superpose 12 Load sample and elute with 1.5 CV (0.6 ml injection, but 300 μl loaded) Conc 8.76 mg/ml (8.76*1.908=16.72)</td>
</tr>
</tbody>
</table>
Appendix B  –  BD-NDPSase methods

B1.  Preparation of rat anti-Bd-NDPSase serum

Rat antisera were developed using a modification of the procedure by Larsson and Nilsson[81]. Ten μL of Bd-NDPSase at 9.3 mg/mL was placed onto each of two one-cm² coupons of sterile nitrocellulose paper and allowed to air dry. Each nitrocellulose coupon was then surgically implanted into the peritoneal cavity of a Sprague Dawley rat. The procedure was repeated on days 10, 20 and 30 with similarly prepared coupons. On day 40, rats were exsanguinated via heart puncture. The resulting sera was stored at -20 °C.

B2.  Immunoelectron microscopy of Bd-NDPSase

A 100 mL *Bdellovibrio bacteriovorus* HD100 liquid culture incubated for 7 days at 30° C was filtered sequentially with 0.80 µm and 0.45 µm filters. The filtrate was centrifuged at 20,000 g for 30 minutes. *Bdellovibrio bacteriovorus* pellets were fixed in 4% formaldehyde in 0.1 M sodium cacodylate buffer with 3% sucrose and 3 mM CaCl₂[82]. Fixed bacteria were then cryopreserved in 2.3 M sucrose in 20% polyvinylpyrrolidone (Sigma). The bacteria were then frozen in liquid nitrogen. Ultra-thin sections were cut with a Leica UCT microtome and the sections were placed on 200 mesh nickel formvar coated grids. Grids were floated section side down on drops of primary antibody diluted in PBS with 10% fetal bovine serum overnight at 4 °C. Primary antibody was detected with 12 nM goat anti-rat secondary gold antibody (Jackson ImmunoResearch, Inc. West Grove, Pennsylvania) diluted 1:20 in PBS for 1 hour at room temperature. Final contrasting of the sections was
done by incubating them in 2% methyl cellulose (Sigma) and 0.3% uranyl acetate (Ted Pella) for 10 minutes at 4°C. All sections were viewed on a Philips CM 120 TEM at 80 kV, equipped with a Gatan Orius SC 1000 digital camera.

B3. **Structural and sequence alignment**

Structural alignments were performed using PDBeFold[83]. The structural homologues were compared to target proteins using PyMOL[46] and LigPlot[84]. The ENDscript[85] web server was used to display and analyze structural alignments. Cavity and dimer interface surface areas were computed using PISA[47]. The amino acid sequences of the structural homologues were aligned using ClustalW[86] and visualized using ESPript[87].
Appendix C  –  RenU methods

C1. Induction of PknG expression and Western blot

Strains were grown to an OD\textsubscript{600} of 2, pelleted and three quarters of the medium were removed. Cells were resuspended in the remaining medium and supplemented with 10 mM inducers. Cultures were incubated at 37°C for 30 minutes, unless otherwise stated, with gentle agitation. The induction was stopped by placing the cultures on ice. Preparations of mycobacterial cell extracts were done as previously described [88]. Briefly, cells were washed three times in phosphate buffered saline (PBS) plus protease inhibitors (Roche), and disrupted by sonication on ice. Cell lysates were clarified by centrifugation (20,000 r.p.m., 20 min, 4°C), treated with SDS sample buffer, and heated at 95°C for 10 minutes. SDS-PAGE was performed using the Bio-rad Protean III system. Proteins were separated on 15 % acrylamide gels and transferred onto PVDF membranes. Western Blot was done using standard procedures, using polyclonal anti-PknG [54], anti-L13 (Josman LLC, Napa, CA) antibodies, or a monoclonal anti-DivIVA (F126-2) [88] antibody. Secondary antibodies coupled to horseradish peroxidase were visualized by chemiluminescence (GE Healthcare Life Sciences).

C2. Bacterial strains and growth media

\textit{M. tuberculosis} H37Rv, \textit{M. bovis} BCG Pasteur, and \textit{M. smegmatis} mc\textsuperscript{2}155 (American Type Culture Collection) were used as parental strains. Mycobacterial strains were grown at 37°C in 7H10 or 7H9 with appropriate supplements and antibiotics. Kanamycin and
hygromycin were used at 50 and 75 µg/ml, respectively. Biofilm growth was done as previously described [89]. For quantitation, a syringe connected to a sterile needle was used to remove the liquid medium and planktonic cells beneath the films. The biomass was harvested and growth estimated through determination of total protein by Bradford method.

C3. Intracellular survival of *M. tuberculosis* strains

Macrophages, generated as described in Extended Experimental Procedures, were seeded in 12-well tissue culture plates (BD Biosciences, San Jose, CA) and let adhere overnight (37°C, 10% CO₂) prior to infection. *Mtb* strains were grown to saturation and infections were performed at MOI 50:1 for 3 hours. Infected macrophages were washed with warm PBS and incubated for 45 minutes with 200 µg/ml amikacin to kill extracellular bacteria, and exchanged into fresh DMEM. At 0 or 72 hours of incubation at 37°C and 10% CO₂, infected macrophages were processed for CFU assays by washing 3 times with PBS, followed by lysis of the macrophages by 0.05% SDS for 5 minutes. Supernatants were harvested, vortexed thoroughly, and plated in triplicate in ten-fold dilutions onto 7H10-OADC agar. Plates were incubated at 37°C for 4 to 5 weeks before CFUs were counted.

C4. Quantification of NADH/NAD and FAD

Growing *M. smegmatis* cultures (OD~2) were treated with 1mM H₂O₂ for 1 hour to induce oxidative stress before determining levels of these nucleoside diphosphate derivatives. Concentrations of NADH and NAD⁺ were measured as previously described [90, 91], calculated from a standard curve of NADH (Sigma N6660-15VL) and standardized to total protein (mg). FAD levels were determined by a colorimetric method using a kit (K357-100) from Biovision, Inc. (Milpitas, CA). All samples and standards were analyzed in triplicates following the supplier’s instruction. FAD, extracted from cell
lysates by perchloric acid, was added to a reaction in which it functions as a cofactor required for an oxidase, which catalyzes the formation of color-generating products. FAD levels were measured through optical absorbance at 570 nm, calculated from a standard curve of FAD, and standardized to total protein (mg).

C5. Intracellular localization of *M. tuberculosis* strains

Macrophage infections and lysosomal trafficking assays were adapted from previously published methods [54]. Macrophages were generated by incubating bone marrow monocytes from C57BL/6 mice for 7 days in high-glucose Dulbecco’s Modified Eagle Medium containing 25% L-929 conditioned medium, 1% penicillin, 1% streptomycin, 4.5 g/L glucose, 4 mM L-glutamine, 15% heat-inactivated fetal calf serum, and 0.02 mg/L macrophage-stimulating growth factor (Sigma Aldrich) at 37°C and 10% CO₂. Macrophages were seeded on MatTek glass bottom 14 mm microwell dishes with coverslip No. 1.5 (MatTek, Ashland, MA) and allowed to adhere for 2 hours (37°C, 10% CO₂) prior to infection. *Mtb* strains (H37Rv, *MtbΔpknG*, *MtbΔpknG/pknG*, *MtbΔrenU*, and *Mtb.L13(T11A)*) were grown to saturation and stained with 0.1 mg/ml FLUOS for 15 minutes. Infections were performed at MOI 50:1 (bacillus:macrophage) for 1 hour at 37°C and 10% CO₂. Infected macrophages were washed 3 times with warm PBS to remove extracellular bacteria, followed by 16-hour chase. After the chase period, lysosomes were labeled for 30 minutes with 1 μM neutral red (Invitrogen) [92], and fixed with 4% formaldehyde for 20 minutes. Slides were mounted in ProLong Gold antifade reagent (Invitrogen). Trafficking was analyzed on a Zeiss LSM510 confocal microscope using LSM510 REL3.5 software provided by the manufacturer. Triplicates of 50 events were recorded and analyzed for each condition from triplicate slides.
C6. Kinase Phosphorylation Assays

*In vitro* phosphorylation activity was assayed as previously described [54], using purified PknG (0.5 µg) or cell lysates to provide kinase activity. The kinase reaction was carried out for 30 minutes at 37°C in 20 µl buffer (10 mM HEPES, 2 mM DTT, 0.4 mM MnCl₂, pH 7.5) containing 10 µCi of [γ-³²P]-ATP (3,000 Ci/nmol, PerkinElmer) and 1.5 µg substrate (purified L13 or RenU). Inhibition of PknG kinase activity was done by preincubation for 15 minutes in the presence of 5 mM AX20017. Reactions were terminated by the addition of SDS-sample buffer and boiled at 95°C for 10 minutes. Proteins were separated on 15% polyacrylamide SDS-PAGE gels and transferred onto PVDF membranes, followed by autoradiography over 6 days. Screens were read on a Storm 820 PhosphorImager and analyzed with ImageQuant software (GE Healthcare Life Sciences).

C7. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5.0f software (La Jolla, CA) and Matlab (Natick, MA). Student’s two-tailed t-test was used to analyze the statistical significance of differences between groups.
Appendix D  –  Nud1p Methods

D1.  Inhibitor screening

The substrate determination assay detailed in section A1.1 was modified to employ the Malachite Green assay (Cayman Chemicals) to detect the release of phosphate following the Nudix catalyzed hydrolysis and phosphatase catalyzed phosphate release. Malachite Green’s absorption characteristics (OD$_{640}$) are better suited for use with multi-well plate reading instruments. A reaction mixture containing 200 nM Nud1p, 10 uM inhibitor, 10 uM CoA, and 20 U/ul CIP in Reaction Buffer (50/5 Tris/MgCl$_2$ pH 8.4) was incubated for 20 minutes before measuring phosphate release. The average optical absorbance of control lanes (no inhibitor present) was calculated and subtracted from the absorbance read from each inhibitor tested. Data were normalized by the maximum value and plotted as a heat map using Matlab (Figure 33).
Figure 33. Nud1p NCC inhibitor assay
Relative inhibition values were colored in an RGB spectrum from 0 (red) to 100 (blue) percent inhibition. Heat map representation of relative Nud1p hydrolase inhibition by compounds in the NIH Clinical Collection drug screen. The screen consisting of 6 96-well plates has been arranged horizontally from top-left to bottom-right, control lanes have been excluded from the heat map. Blank squares denote wells in Plate VI for which inhibition trials were not conducted.

The lead NCC compounds exhibiting the highest relative Nud1p hydrolase inhibition (Table 5) were purchased from Sequoia Research Products, Ltd (Pangbourne, UK). Their dissociation constants ($K_d$) were calculated by conducting a differential scanning fluorimetry (DSF) assay as previously described [93]. Nud1p’s thermostability at a 10 µM concentration in a 20 µl reaction volume was measured in the presence of individual lead compounds at a 500 µM concentration. Dissociation constants were calculated from the DSF melt curves as described by Pantoliano [94].
References


Curriculum Vitae

Andres Hernandez
2015

Educational History:

Ph.D. expected 2015 Biomedical Engineering Johns Hopkins Univ.
Mentors: Mario Amzel, PhD School of Medicine
Sandra Gabelli, PhD

B.S. 2009 Biomedical engineering University of Houston

Other Professional Experience

Graduate Student 2009-2015 Johns Hopkins Univ.

Developed a method to characterize proteins using incomplete factorial screens
Conceptualized and implemented an assay to study a key signaling pathway in M. tuberculosis
Developed the ThermoQ software and HotProteins.com website to study protein thermostability
Implemented an assay to estimate substrate $K_d$ from binding induced thermostability changes

Intern 2014 Quidel Inc.

Designed the process flow and proved the effectiveness of a Norovirus diagnostic kit
Designed the process flow of an influenza diagnostic kit

Research Tech. 2009 Methodist Hospital RI

Managed the data servers of the Next Generation Sequencing Core

Intern 2008 Methodist Hospital RI

Developed an assay to quantify protein quality and identify ligands
Led to a $199,725 CPRIT grant to screen for estrogen receptor ligands

Intern 2006-2007 Alliance for Grad. Edu. And the Professoriate

Developed an immersive visualization and particle tracking algorithm for blood flow simulations
Identified novel blood flow patterns to help physicians design interventional devices

Student/Volunteer 2005-2008 University of Houston

Designed and built a nitinol-woven self-expandable aortic stent. Proved functionality and integrity through fluid dynamic simulations, stress tests, and corrosion tests
Designed and built a battery-powered ECG selected for display at the College of Engineering

Created a mathematical definition of a vortex. Dramatically reduced the computational time for vortex identification and analysis in fluid dynamics simulations

Scholarships and fellowships

2011 National Sci. Foundation Graduate Research Fellowship Johns Hopkins Univ.
Secured $126,000 award to pursue my own original research

2007 Provost Undergraduate Research Scholarship University of Houston
Secured $1000 to conduct a computational research project

2007 Julie Ryan Academic Scholarship University of Houston
Recognized for high academic achievement

2006 University Student Fund Scholarship University of Houston
Recognized for high academic achievement

**Honors and awards**

- 2014: On the Spot Award, *Quidel Inc.* Recognized for significant contributions to the development of an Influenza molecular diagnostics product
- 2009: Outstanding Senior Honors Thesis, *University of Houston* Recognized as one of the best thesis written in the Honors College
- 2007: Tau Beta Pi Engineering Honor Society, *University of Houston* Top 12% of engineering students
- 2006: Order of Omega Honor Society, *University of Houston* Top 3% of fraternities and sororities
- 2005: Sound Mind Award, Sigma Phi Epsilon Fraternity, *University of Texas SA* Recognized for high academic achievement and tutoring

**Peer Reviewed Publications**


**Other Publications**


**Conference Proceedings**


Inventions, Patents, and Copyrights
2015 DSF Guided Refolding as a Novel Method of Protein Production (patent pending)

Service and Leadership
2010-2013 Diversity Leadership Council Johns Hopkins University
Appointed three times by President Ronald Daniels
Worked with senior administrators to advise the President on issues of diversity and inclusion
Co-founded the Latino Alliance
Co-founded the Diversity Innovation Grants Program
2012-2013 Coordinator of Crystal Club Johns Hopkins University
Organized an X-ray crystallography speaker series
2011 Graduate Student Association Johns Hopkins University
Student Assistant Program student representative
2008-2009 Coordinator of Engineering Futures University of Houston
Organized a professional development speaker series
2010 Coordinator of Second Look Weekend Johns Hopkins University
Coordinated the first inclusive recruitment weekend for minority medical and graduate students
2009 Tau Beta Pi District 10 Conference Tau Beta Pi
Attended a leadership training conference
2005 Carlson Leadership Academy Sigma Phi Epsilon
Attended an immersive leadership training program
2005 Student Government Association University of Texas SA
Co-founder of Freshmen Involvement Raising Excellence
Created an organization design to introduce freshmen to local and school government