STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE
HUMAN TRIBBLES HOMOLOGUE 2 PSEUDOKINASE

by

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Abstract

The Tribbles Homologues are a family of three eukaryotic pseudokinases (Trb1, Trb2, Trb3) that act as allosteric inhibitors and regulatory scaffold sites in pathways governing adipogenesis, cell proliferation and insulin signaling. The Tribbles Homologues have the same overall tertiary structure of the eukaryotic protein kinase domain, but lack multiple residues necessary to catalysis in the nucleotide-binding P-loop and the Mg\(^{2+}\)-coordinating DFG motif. Trb1 has been shown conclusively to be incapable of binding ATP, whereas a recent study presents evidence that Trb2 autophosphorylates independently of Mg\(^{2+}\) \textit{in vitro}. This finding is surprising given the high degree of sequence similarity between the two proteins (71%), and suggests unique nucleotide binding and phosphotransfer mechanisms. The goal of this project was to investigate whether Trb2 possesses kinase activity or not and determine its structural basis. A method for the high-yield recombinant expression and purification of stable Trb2 was developed. Trb2 nucleotide binding and autophosphorylation could not be detected across multiple experimental approaches, including thermal shift assays, MANT-ATP fluorescence, radiolabeled phosphate incorporation, and nonspecific ATPase activity assays. Further characterization also revealed that Trb2 forms homomultimers with possible functional consequences, and extensive crystallization screening has yielded multiple promising conditions that could produce diffraction-quality crystals with further optimization. This project explores the difficulties in functionally characterizing putatively active pseudokinases, and proposes a structural basis for the conserved pseudokinase features of the Tribbles homologues.
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- Greg Hicks
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List of Abbreviations

A$_{280}$ absorption at 280 nm
AML acute myeloid leukemia
AUC analytical ultracentrifugation
C1BD COP1 Binding Domain
CaM Ca$^{2+}$/calmodulin-dependent
CASK Ca$^{2+}$/calmodulin activated Ser-Thr kinase
CD circular dichroism
CTR C-terminal region
DSF differential scanning fluorimetry
DTT dithiothreitol
E. coli Escherichia coli
EDTA ethylenediaminetetraacetic acid
ePKD eukaryotic protein kinase domain
FPLC fast protein liquid chromatography
HDVD hanging drop vapour diffusion
IMAC immobilized metal affinity chromatography
IPTG isopropyl-β-D-1-thiogalactopyranoside
MBP maltose binding protein
Ni$^{2+}$-NTA nickel-nitrilotriacetic acid
NTR N-terminal region
OD$_{600}$ Optical density at 600 nm
PCR polymerase chain reaction
P$_i$ inorganic phosphate
PKA cAMP-dependent protein kinase
RFU Relative Fluorescent Units
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDVD sitting drop vapour diffusion
TEV tobacco etch virus
Tris Trisaminomethane
TSA thermal shift assay
Chapter 1

Introduction

1.1 Eukaryotic Protein Kinases and Pseudokinases

The eukaryotic protein kinases (ePKs) are a large superfamily of proteins related by a common catalytic domain. The ePKs are encoded by over 500 unique genes, making up ~1.7% of the entire human genome.\(^1\) They catalyze the post-translational addition of phosphate groups donated by nucleotides (usually ATP) to hydroxyl amino acid side chains (serine, threonine, tyrosine), which acts as a substrate-specific molecular switching event. Protein phosphorylation is a widespread signaling mechanism, with protein kinases playing a role in virtually all cellular processes including proliferation, immunity, metabolism and stress response. An estimated 2/3 of the human proteome is phosphorylated on one or more residues, representing a staggering number of unique targets that ePKs need to specifically recognize. Substrate recognition is controlled in part by accessory protein-binding domains, but the binding of the appropriate phosphotransfer site is ultimately determined by specific motifs in the catalytic domain proper.\(^2\) Research elaborating on the functions of ePKs in signaling networks beyond simple kinase-substrate relationships has begun to show that the structural features that make the kinases potent catalytic signalers also contribute to non-catalytic signaling through allostery, scaffold complexes, and even competitive inhibition.\(^3\) These non-catalytic properties provides a rationale for the existence of ePK domains that lack phosphotransfer activity, but still serve conserved roles in signaling.

The pseudokinases were identified when the first genome-wide survey of protein kinases showed that up to 10% of genes predicted to have the ePK tertiary fold lacked one or more
residues essential to kinase activity.¹ The motifs in pseudokinases that deviate from canonical kinase sequence are typically conserved across species variants, indicating that normal kinase activity has been selected against in favour of non-catalytic function. The non-catalytic functions of pseudokinases are as central to cellular signaling as their active counterparts, and their lack of kinase activity is often crucial to their biological role. For example, the V617F mutation in the JAK2 pseudokinase restores kinase activity, but also promotes the development of myoproliferative blood cancers. The catalytic shortcomings of pseudokinases are not the products of atrophied catalysis, but conserved adaptations in their own right. Pseudokinase evolution is convergent – changes to any aspect of the ePK catalytic motifs can result in compromised function. The following section will discuss the structural basis of ePK catalysis, and the different types of deviations from canonical sequence that can result in pseudokinases.

1.1.1 Eukaryotic Protein Kinase Structure and the Basis of Pseudokinase Identity

Pseudokinases retain the general tertiary fold of the ePK domain, consisting of two globular regions subdivided into 12 subdomains: an N-terminal lobe comprised mostly of β-sheets (Subdomains I – IV) connected by a flexible hinge region (Subdomain V) to a C-terminal lobe that is mostly α-helical (Subdomains VIA-XI) (Figure 1.1). When an ePK domain is inactive, the two lobes move freely relative to one another in an inactive “open” conformation. In order for productive catalysis to occur, most ePKs must bind a nucleotide phosphate donor (usually ATP), metallic cofactors (Mg²⁺ or Mn²⁺), and the appropriate recognition sequence of its substrate. Activation either by post-translational modifications or allosteric binding is also often necessary for the reaction to proceed. When these conditions are met, the N and C-terminal lobes come together to form the catalytic cleft in an active “closed” conformation. The catalytic cleft facilitates the nucleophilic attack of the substrate hydroxyl group by the donor phosphate. Once
phosphorylation is complete, the lobes must separate and release the dephosphorylated nucleotide and the phosphorylated substrate in order to begin the next round of catalysis. Some pseudokinases are locked constitutively in a particular conformation, while others still undergo an open-closed transition in response to ATP or allosteric interactions, like STRAD. Activation signals vary amongst the ePKs, depending on specific binding partners or post-translational modifications. It is therefore difficult to predict based on sequence alone whether an ePK, pseudokinase or otherwise, is able to undergo the close/open transition. Pseudokinase annotation is usually based on differences in the residues directly involved in nucleotide binding, cofactor coordination, or the catalytic transfer of phosphate. As is the norm when discussing ePK structure, canonical active kinase residue positions will be referred to relative to their positions in protein kinase A.
Figure 1.1.1 Structure and Conserved Catalytic Motifs of PKA

(A) The mostly-β N-lobe and the all-α C-lobe are connected by a flexible hinge region. ATP is coordinated by a conserved lysine Mg$^{2+}$ bound to the DFG loop, and the flexible glycine-rich loop. When ATP is bound, the two lobes come together in a closed, active conformation. Figure modified from Taylor and Kornev (2011).

(B) Linear architecture of the eukaryotic protein kinase domain, showing the relative positions of conserved catalytic residues, secondary structure elements, and the 12 subdomains.

The nucleotide substrate of an active kinase in the active bound state is precisely coordinated by multiple motifs and cofactors, as the γ-phosphate to be transferred needs to precisely aligned for catalysis to occur. Many pseudokinases still possess the catalytic machinery and substrate affinity of active kinases, but are unable to bind ATP. Lys72 in subdomain II coordinates the α and β phosphates of ATP, and is held in place by a salt bridge interaction with
Glu91. Lys72 is critical to nucleotide binding, and is commonly mutated in experiments testing the effects of kinase inactivation. ePK domains that lack lysine at that position typically cannot bind ATP, with some exceptions. The “With No K” Kinase (WNK1) was predicted to be an inactive pseudokinase based on its lack of Lys72, but still displayed catalytic activity in vivo. This illustrates the difficulty in predicting kinase activity based on sequence alone, as differences that may completely inactivate most kinases may be compensated for by less obvious sequence elements.

A glycine-rich loop in subdomain I (GXGXXG), alternatively called the “P-loop” also contributes to ATP binding. The P-loop is flexible due to its high glycine content, allowing it to coordinate the α and β phosphates through backbone hydrogen bonding interactions. A lack of glycines or the prevalence of bulky residues in the P-loop can limit the loop’s flexibility and by extension its affinity for ATP, but pseudokinases with degenerate P-loops usually feature at least one other source of nucleotide-binding deficiency.

Kinase activity can also be disrupted by an inability to bind metal cofactors. Nearly all active kinases bind Mg$^{2+}$ or Mn$^{2+}$ as cofactors, which further coordinate the nucleotide phosphates. Two cations are bound by the DFG (184-186) loop in subdomain VII and Lys168 in subdomain IV via electrostatic interactions. Pseudokinases that lack a complete DFG loop cannot bind Mg/Mn$^{2+}$, reducing their affinity for ATP and preventing the proper catalytic coordination of the γ phosphate. A noteworthy exception is the CASK-CaM kinase, an active pseudokinase which coordinates the β and γ phosphates through a less efficient cation-independent mechanism whereby the labile phosphates are coordinated exclusively by kinase residues. CASK is in fact inhibited by Mg$^{2+}$, and phosphorylates its effector, neurexin, in
response to synaptic cation efflux.\textsuperscript{12} Although CASK is a unique case, it demonstrates the need to account for the cellular context when identifying putative pseudokinases.

The phenylalanine of the DFG motif acts as a “gatekeeper” residue that prevents ATP from entering the binding pocket prematurely. The position of the gatekeeper phenylalanine is controlled by a variable activation loop between the DFG loop and a conserved APE motif. The activation loop requires one or more activating signals to allow the gatekeeper to exit the binding pocket.\textsuperscript{13} This can occur through allosteric mechanisms, but most protein kinases require phosphorylation of one or more activation loop residues for the transition to occur. Recognition of the activation loop is protein-specific and therefore difficult to predict. It is important to consider that active ePKs assayed for kinase activity \textit{in vitro} may appear inactive if the necessary activating kinases are not present, or that predicted active kinases are in fact constitutively inactive due to an immobile APE motif.

Many pseudokinases bind ATP and metal cofactors like active kinases, but are inactive due to changes to the core catalytic machinery. Once ATP and substrate have bound a kinase in the active conformation, residues on the catalytic loop in subdomain VIB (HRDXXXXN) facilitate phosphate transfer. The aspartate in the catalytic loop is universally conserved among active protein kinases, as it accepts a proton from the substrate residue’s hydroxyl group as an intermediate, allowing it to nucleophilically attack the nucleotide $\gamma$-phosphate. Once phosphotransfer has occurred, the aspartate is deprotonated and the Mg$^{2+}$ coordination complex dissipates. The resultant ADP and phosphorylated protein substrate are then released. The residues flanking the aspartate are more variable throughout the kinome. In active kinases, the histidine (D-2) is present in most active kinases, stabilizing the hydrogen bonding network of the active conformation and the Mg$^{2+}$ coordination geometry of the DFG loop.\textsuperscript{14} Mutations to the
catalytic loop histidine do not eliminate Mg$^{2+}$ binding, but do limit the overall stability of the active conformation. The conserved asparagine (D+4) also contributes to Mg$^{2+}$ binding, but is not considered essential like the DFG loop. The ePK catalytic loop is host to several variations that can modulate catalytic activity, but only the absence of the catalytic aspartate is a strong predictor of pseudokinase identity.

It is ultimately difficult to conclusively identify pseudokinases based exclusively on sequence analysis. Despite the emphasis on “canonical” kinase sequences, the active kinome does display a degree of variation in catalytic residues - for nearly every individual sequence variation ascribed to a predicted inactivity, there is a confirmed kinase that has activity despite it.¹⁰ This diversity is reflected among the pseudokinases: some pseudokinases like VRK3 or the SCYL family have completely degenerate catalytic clefts, whereas others like JAK2 or the SgK pseudokinases only have a handful of residues that differentiate them from active kinases. Whether they are active or not, the fact that multiple ePKs have been selected for reduced activity through multiple selective events illustrates that the domain has been retained for intracellular functions beyond protein phosphorylation.

1.1.2 Non-Catalytic Signaling Functions of Pseudokinases

All pseudokinases maintain the bilobal ePK domain structure regardless of how little the catalytic cleft resembles that of an active kinase. The pseudokinase domain retains the ePK’s capacity for non-catalytic signaling through its capacity for specific binding-site recognition, and the conformational mobility of the N and C-terminal lobes. The following examples illustrate some of the recurring mechanisms by which pseudokinases can exert specific regulatory control over signaling pathways.
Figure 1.2 Examples of Pseudokinase Regulatory Functions

Pseudokinases serve pivotal regulatory roles through non-catalytic binding. (A) The STRAD pseudokinase is stabilized in the closed conformation by MO25, which then form a complex to activate the LKB1 kinase. (B) The HER3 pseudokinase binds and activates EGFR at an increased rate when bound to ATP, adding an additional level of energy-intermediate sensitivity to EGFR signaling. (C) The KSR pseudokinase forms a multiunit scaffold that recruits the downstream components of the Ras-activated MAPK cascade to the membrane and coordinates Raf/Mek/MAPK phosphorylation. Modified schematic from Boudeau et al. (2006). Some predicted pseudokinases exhibit abnormal kinase activity. (D) The CASK kinase lacks a canonical DFG loop and is inhibited by divalent cations, including Mg$^{2+}$. CASK phosphorylates neurexin in response to synaptic cation flux, regulating synaptic junction formation in a cation-dependent manner.
1.1.2.1 Allosteric Regulation

Many pseudokinases take advantage of the fact that most protein kinases are also the substrates of other kinases, and regulate kinase activity by binding in a substrate-like manner. The pseudokinase STRAD lacks most of the canonical catalytic residues, but is stabilized in the active conformation through interaction with the MO25 scaffold protein. The “active” STRAD then binds the LKB1 kinase in a substrate-like manner, inducing an allosteric shift that activates LKB1 phosphorylation of AMPK (Figure 1.2A). STRAD functions to take advantage of the mobility of the N and C-terminal globes and the protein substrate recognition properties of the catalytic cleft region to specifically regulate LKB1.

Some pseudokinases bind nucleotides in a non-catalytic manner, acting as allosteric regulators sensitive to cellular energy levels. Human epidermal growth factor receptor 3 (HER3) is an inactive tyrosine kinase that activates EGFR and HER2 through formation of a heterodimer. HER3’s affinity for EGFR is increased when HER3 binds ATP, boosting activation of the epidermal growth factor signaling pathway (Figure 1.2B). Changes in binding affinity and signaling function in response to nucleotide availability can link pseudokinase regulation directly to the availability of metabolic energy intermediates.

1.1.2.2 Protein Scaffolding

The most widely researched element of pseudokinase function is their role as protein scaffolds. Scaffold proteins act as coordination centres for multiprotein complexes, and can promote the activity of a signaling pathway by co-localizing signal enzymes, or inhibit it by sequestering key enzymes from active complexes. For example, the KSR pseudokinase facilitates the initiation of the Ras/Raf/Mek/MAPK signal cascade by recruiting MAPK and Mek
to the inner membrane, allowing Ras-activated Raf to phosphorylate the latter (Figure 1.2C). KSR contributes no signaling activity itself, but facilitates the rapid transmission of information through the pathway by coordinating its active kinase components.

1.1.3 Active Pseudokinases

Pseudokinase deviate from the canonical catalytic motif sequences of active kinases, and many have been shown to possess signaling functions independent of phosphotransfer activity. Despite this, a growing body of evidence has shown that many pseudokinases display some weak kinase activity. These findings are contentious when based solely on in vitro evidence. When the target kinase activity is weak, the risk of false positives is high due to low concentrations of contaminating kinases left over from purification. Additionally, pseudokinases that have been rendered inactive by low nucleotide affinity may show activity in the presence of artificially high concentrations that do not reflect in vivo levels. Rigorous confirmation through mutagenic analysis and in vivo experiments is necessary to confidently assess the activity of a pseudokinase.

Two examples of pseudokinases with weak but biologically relevant kinase activity are the CASK-CaM kinase and WNK1. The misleadingly-named “With No K” Kinase (WNK1) lacks the key catalytic lysine on the β3 strand (Lys72 on PKA), but has been shown to inhibit the sodium transport regulator WNK4 via phosphorylation.\(^{16}\) The WNK1 crystal structure reveals that a different lysine residue on the β2 strand extends into the catalytic site and serves the same function.\(^{8}\) The activity of WNK1 demonstrates that the canonical kinase catalytic residues are imperfect predictors of kinase activity, and that the possibility of alternative catalytic mechanism should be considered when annotating pseudokinase function.

The CASK-CaM kinase is even more unusual, as it displays kinase activity in vivo independently of metallic cofactors. CasK-CAM lacks a DFG loop and is in fact inhibited by
divalent cations. The ATP β and γ phosphates are coordinated through a less efficient cation-independent mechanism. CasK has been shown to phosphorylate itself and neurexin, a synaptic junction regulator, in response to synaptic cation flux (Figure 1.2D). CasK’s compromised kinase activity represents an adaptive tradeoff, whereby catalytic potency was lost in favour of a specific regulatory response to synaptic function. Although pseudokinases with known function have been shown to not require catalytic activity, both WNK1 and CasK illustrate the possibility that diminished kinase activity could represent a conserved adaptation of phosphorylation-dependent signaling pathways.

The existence of predicted pseudokinases that display catalytic activity introduces some ambiguities into ePK nomenclature. Pseudokinase activity likely involves alternative catalytic mechanisms, which would match the definition criteria for atypical kinases. However, to call many active pseudokinases atypical kinases would be misleading as it would incorrectly imply that ambiguously active pseudokinases that show weak evidence of activity only in vitro have biologically relevant activity in vivo. Furthermore, the activity of many pseudokinases with confirmed in vivo activity such as HER3 or the JAK2 pseudokinase domain are less crucial to their signaling function than their capacities for nucleotide binding and protein-protein interactions. To deem them atypical kinases would once again over-represent the importance of their capacity for phosphotransfer. Therefore, for the purposes of this project we have proceeded with the definition of pseudokinases proposed by Murphy et al. (2015) as: an ePK that is predicted to display compromised kinase activity due to one or more missing catalytic residues.

1.2 The Tribbles Homologue Pseudokinase Family

The Tribbles (Trb) homologues are a family of eukaryotic pseudokinases named for their sequence homology with Tribbles, a pseudokinase that regulates embryonic mitosis in
*Drosophila melanogaster*. The name “Tribbles” derives from the observation that mesodermal cells in tribble-negative embryos exhibit uninhibited proliferation in a manner reminiscent of the rapidly reproducing alien Tribbles from *Star Trek*.\(^\text{17}\) The three homologues – Trb1, Trb2 and Trb3 – were first identified in vertebrates through a functional genome screen of inflammation response signaling networks.\(^\text{18}\) The human Tribbles homologues are of general interest due to their roles in several disease states. Trb1 and Trb2 have been shown to be tumor promoters when overexpressed, contributing particularly to the development of acute myeloid leukemia (AML).\(^\text{19}\) Trb2 and Trb3 have been shown to inhibit components of the insulin signaling response pathways, and naturally occurring Trb3 Gln84Arg polymorphism has been shown to promote insulin insensitivity and cardiovascular disease.\(^\text{20}\) Curiously, elevated levels of anti-Trb2 antibodies have been found in patients with cataplexy-narcolepsy, suggesting it may be an autoantigen involved in the development of the condition.\(^\text{21}\) No mechanism for this has been identified, suggesting a hereto unknown neurological function of Trb2. The Tribbles homologues have potential as therapeutic targets for several diseases, but a better understanding of the structural basis of their signaling functions is necessary for the rational design of inhibitors.

The Tribbles homologues share high sequence identity with the canonical two-lobed eukaryotic protein kinase domain, but feature key variations specific to their regulatory functions. They have been designated as pseudokinases based on differences in multiple key catalytic residues. The Tribbles homologues all have the critical ATP-binding Lys72 and catalytic Asp166 equivalents, but are predicted to be unable to coordinate Mg\(^{2+}\) due to an absent DFG loop motif. The region corresponding to the GXGXXG loop consists of fewer flexible residues, which would limit the Trbs’ ability to bind nucleotides. The catalytic aspartate is present, but the flanking catalytic loop histidine and asparagines responsible for stabilizing the
active conformation and coordinating Mg$^{2+}$ are absent from all three Tribbles homologues. The differences in catalytic residues between the Tribbles homologues and PKA are summarized in Figure 1.3. Based on sequence analysis, the Tribbles homologues are predicted to have a limited capacity for nucleotide binding, Mg$^{2+}$ coordination, and kinase activity. The Trb1 crystal structure and bioinformatic analysis of the Tribbles homologues support this prediction, and provide clues as to how their signaling functions are regulated \textit{in vivo}.

![Trb1 PDB: 5ECM](image)

<table>
<thead>
<tr>
<th>PKA</th>
<th>GTGSFG</th>
<th>DFG</th>
<th>HRDLKLRN</th>
</tr>
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<tbody>
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<td>Trb1</td>
<td>LPLAER</td>
<td>SLE</td>
<td>LGDLKLRK</td>
</tr>
<tr>
<td>Trb2</td>
<td>LEPEEG</td>
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</tr>
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<td>Trb3</td>
<td>LEPEEG</td>
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<tr>
<th>Active Kinase Function</th>
<th>ATP binding</th>
<th>Mg$^{2+}$ coordination</th>
<th>Catalytic Loop</th>
</tr>
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</table>

**Figure 1.3 Degenerate Catalytic Residues of the Human Tribbles Homologues**

Crystal structure of the degenerate Trb1 catalytic cleft (PDB:5ECM) and local alignments of the corresponding human Tribbles homologues’ sequences relative to the canonical active kinase, PKA.
1.2.1 Structural Features of the Tribbles Homologues

All of the Tribbles homologues feature a disordered N-terminal region (NTR), a eukaryotic pseudokinase domain (PKD), and a C-terminal region (CTR) that includes a COP1-binding domain (C1BD). To date, only one crystal structure of Tribbles homologues (Trb1) has been solved. As such, most of what is known about Trb2 and Trb3’s structure is based on bioinformatic prediction, or inferred by similarity to Trb1.

1.2.1.1 N-Terminal Region

The N-terminal region (NTR) is the most variable region of the Tribbles homologues and has been predicted to affect protein turnover and localization. Secondary structure predictions indicate that the NTR is intrinsically disordered, primarily due to its high concentration of PEST sequences (Figure 1.4A). PEST sequences are regions containing high concentrations of proline, glutamate, serine and threonine residues that have been found to promote proteolytic degradation.\(^ {22}\) This would explain in part the relatively low half-lives of Tribbles homologues, as well as the improved expression of truncations lacking the NTR.\(^ {19}\) Additionally, PEST-mediated degradation has been shown to be phosphorylation-dependent in some instances.\(^ {23,24}\) Although no specific phosphorylation sites have been identified on the Tribbles homologues, the possibility of phosphorylation-enhanced PEST turnover regulation should be considered.

The Tribbles homologue NTR has also been connected to subcellular localization. Trb1 and Trb3 localize to the nucleus in HeLa cells, whereas Trb2 is found in the cytoplasm.\(^ {25,26}\) Trb1 and Trb3 share more sequence similarity in the conserved region of the NTR region flanked by the two PEST motifs than either do with Trb2. The conserved \([K/R]_2[D/E]_2[D/E]_NTR sequence has been identified as a probable localization signal.\(^ {27}\) Although it has not been confirmed
experimentally, the sequence features of the NTR suggest a role in subcellular localization as well as turnover regulation.

![Sequence alignment](image)

Figure 1.4 Structural Features of the Human Tribbles Homologues

(A) Sequence alignment of the human Tribbles homologues’ NTR segments showing the conserved PEST residues (grey) and putative localization sequence \([K/R]_{2}[D/E][D/E]\) (purple). (B) Sequence alignment of human Tribbles homologues’ CTR, showing the MEK binding (blue), COP1 binding (green) motifs. (C) Crystal structure of Trb1 (PDB:5ECM) shows the bilobal pseudokinase domain (red) in complex with the CTR (yellow). The region connecting the CTR to the C-terminal lobe of the PKD is disordered, and is not shown in the crystal structure. Sequences aligned by ClustalW and visualized in UGENE.\(^{28,29}\)
1.2.1.2 C-Terminal Region and the COP1 Binding Domain

The C-terminal region (CTR) of the Tribbles homologues contains two conserved motifs necessary to protein-protein interactions (Figure 1.4B). The conserved “ILLHPW(F/L)” motif binds MEK components of the mitogen-activated protein kinase (MAPK) cascade and is essential to the formation of Trb-coordinated MAPK scaffold complexes. The MEK-binding motif is tightly conserved between the Tribbles homologues, but all three bind different MEK proteins. It is therefore likely that a complementary region on the PKD is responsible for MEK-binding specificity. The “DQIVP(E/D)” residues are designated as the COP1-binding domain (C1BD), as they are essential to recruitment of the E3 ubiquitin ligase. The crystal structure of Trb1 also shows that the C-terminal COP1-binding domain closely associates with the pseudokinase domain. The VPE component of the C1BD fits into a groove between the β4 strand and αC helix, in a manner similar to other kinase regulatory domains.

1.2.1.3 Pseudokinase Domain

The Trb1 crystal structure shows the canonical bi-lobal ePK domain with a number of deviations in the N-terminal lobe that prevent ATP binding. The region between β strands 1 and 2 that corresponds to the glycine-rich loop in PKA is contracted by a kink introduced by Pro98, preventing backbone interactions with the β- and γ-phosphates. The αC helix adjacent to β3 is bent and truncated, preventing it from forming a wall of the nucleotide binding pocket. In addition to forming a degenerate binding pocket, the Trb1 SLE motif in place of the canonical DFG forms several interactions that prevent nucleotide binding. Most obviously, the polar serine (Ser225) would be less effective at coordinating metallic cofactors than the anionic aspartate. The SLE leucine (Leu226) is held in a position analogous to the gatekeeper phenylalanine by a pocket of conserved hydrophobic resides. This conformation of Leu226 is further stabilized by a
hydrogen bonding between its backbone carbonyl and Lys120 in subdomain III. The immobile Leu226 gatekeeper likely limits the ability of nucleotides to enter the catalytic cleft. The sequence features contributing to the degenerate nucleotide binding pocket seen in the Trb1 crystal structure are conserved in Trb2 and Trb3.

Together, these deviations from the canonical kinase structure render Trb1 incapable of binding nucleotides, and eliminate the possibility of phosphotransfer activity. This was confirmed further by a fluorophore thermal shift assay (TSA), which showed no evidence of interactions between Trb1, Mg$^{2+}$, or nucleotides. Based on its structure and biophysical assays, it can be concluded that Trb1 is not an active kinase and does not bind ATP. Given that Trb2 and Trb3 share these features, it would appear unlikely that either is able to bind ATP or display kinase activity.

1.2.2 Functions of the Tribbles Homologs

The Tribbles homologues are a functionally diverse family of proteins, acting as non-catalytic regulators in pathways controlling differentiation, lipid metabolism, inflammation, cell cycle arrest, and insulin response. A few of their better-understood functions are summarized below.

1.2.2.1 Proteasomal Degradation of C/EBPα and ACC1

The Tribbles homologues target regulatory factors in lipid metabolism for proteasomal degradation. The C1BD binds COP1, an E3 ubiquitin ligase. When acting with the minimum components of a ubiquitination reaction, COP1’s primary mammalian substrate is the p53 tumour suppressor. On its own, COP1 regulates p53 turnover but, like other E3 ligases, can be targeted to different ubiquitination substrates by scaffolding intermediaries. All three of the Tribbles homologues, as well as the ancestral Tribbles, have been shown to retarget COP1 to
different ubiquitination substrates: Tribbles to CHOP (C/EBP homologous protein), Trb3 to acetyl-coenzyme A carboxylase (ACC), and both Trb1 and Trb2 to C/EBPα (Figure 1.5A).\textsuperscript{34,35}

The C/EBPα Tribbles recognition a likely binding site on the Trb1 PKD has been identified opposite the degraded catalytic cleft.\textsuperscript{31} This suggests that the PKD is necessary to substrate recruitment, whereas the C1BD is responsible exclusively for recruiting COP1 to the complex. ACC controls the rate-limiting step of fatty acid oxidation, and C/EBPα controls the transcription of factors controlling adipogenesis, among other functions. The Tribbles homologues therefore play a central role in the regulation of lipid metabolism. Overexpression of Trb1 and Trb2 can also cause AML, as C/EBPα is a critical suppressor of myelogenous tumor progression.\textsuperscript{19}

The degenerate kinase catalytic motifs of the Tribbles homologues have been shown to play some role in COP1 ubiquitination. Keeshan \textit{et al.} (2010) showed that Trb2-dependent C/EBPα degradation was completely obstructed when the Trb2 pseudocatalytic loop motif (LRDLKLRK) was mutated to resemble a canonically active Ser/Thr kinase (HRDLKPEN). This suggests that the degraded catalytic motifs of the Tribbles homologues contribute to the structure of unique binding surfaces that are essential to their \textit{in vivo} regulatory function.
1.2.2.2 Akt/PKB Inhibition

The Tribbles homologues negatively regulate Akt/PKB. Akt is a central node of the insulin response signal cascade and its signaling kinase activity is tightly regulated by multiple activating and inactivating phosphorylation sites. The two most crucial activating phosphorylation sites are Thr308 and Ser473, which are phosphorylated by mTORC2 and PDK1, respectively. The Tribbles homologues modulate insulin response in vivo by preventing the activating phosphorylation of Akt at these residues. The Tribbles homologues have been shown to interact directly with Akt, but not with any of its upstream activators. It is therefore possible that the Tribbles homologue PKD acts as a “decoy kinase” by binding the Akt activation site, competitively inhibiting mTORC2 (Figure 1.5B). The potency of Akt inhibition varies between the Trb homologues, with Trb3 displaying a much more pronounced effect on Akt activity than Trb1 and Trb2. This suggests some variability between the Tribbles homologues in the region responsible for Akt recognition and inhibition that has yet to be identified.

1.2.2.3 Inhibition of Mitogen-Activate Protein Kinase-Kinases

The Tribbles homologues modulate mitogen activated protein kinase (MAPK) cascade signaling by interacting with the associated scaffold complex and inhibiting intermediary MAPKK activity (Figure 1.5C). Trb3 binds MEK4, Trb1 binds MEK1 and MEK4, and Trb2 binds MEK7 and MEK1. It has been proposed that the overlapping binding profiles and differential expression of the Tribbles homologues dictates the tissue-specific responses to the associated MAPK signal cascades. The mechanism for Tribbles homologue inhibition of MAPKK phosphorylation is unknown. The conserved ILLHPW(F/L) component of the C1BD participates is essential to MAPKK binding, suggesting a mechanism similar to C/EBPα/ACC recruitment. The MAPKK recognition sequence is tightly conserved between the three Tribbles...
homologues, so specificity for the different MAPKK’s could be conferred by a variable region in the PKD. The Tribbles homologues could therefore act as “decoy kinases” as with Akt, but by mimicking the downstream MAPK substrate rather than the upstream activator kinase.

Figure 1.5 Functional schematics of Tribbles homologue regulatory function

The Tribbles homologues are negative regulators of several intracellular signaling pathways. (A) The COP1 E3 ubiquitin ligase is recruited by the Tribbles homologue C1BD, targeting C/EBPα or ACC bound to the PKD for ubiquitination and subsequent degradation by the proteasome. (B) Trb2 and Trb3 prevent phosphorylation of the Akt activation loop by mTORC2 and PDK1, modulating the downstream insulin response cascade. (C) All three Tribbles homologues interact with intermediary MAPK kinases and inhibit the activating phosphorylation of downstream MAPK effectors.
The Tribbles homologues modulate multiple signaling pathways through coordinated recruitment of binding partners by its PKD and CTR. There is no in vivo evidence that the Tribbles homologues phosphorylate any of their regulatory effectors, which is consistent with their recurring roles as inhibitors of phosphorylation-activated protein kinases. The degraded catalytic features of the PKD have also been shown to be essential to scaffold coordination of the COP1 ubiquitination complex. Considering the above, it would be reasonable to conclude that the Tribbles homologues are constitutively inactive pseudokinases that do not require protein kinase activity to fulfill their roles in cellular signaling. However, recent publications have raised the possibility that one or more of the human Tribbles homologues are capable of ATP-dependent phosphate transfer through a novel catalytic mechanism.40

1.2.3 Trb2 is a Putative Mg\(^{2+}\)-Independent Kinase

Until recently, all three human Tribbles homologues were assumed to be catalytically inactive, as well as incapable of binding nucleotides. A survey of pseudokinases in 2014 by Murphy et al. using a high-throughput fluorescent thermal shift assay (TSA) strategy was able to confirm the weak nucleotide binding capacity of several pseudokinases, but showed no evidence of binding by the Tribbles homologues.41 A more recent study by Bailey et al. (2015) using the same method, conversely, reported that Trb2 displays magnesium (Mg\(^{2+}\))-independent ATP binding.40 Further characterization using radiolabelled \([^{32}\text{P}]\)-\(^\gamma\)-ATP showed that Trb2 autophosphorylates independent of Mg\(^{2+}\) in vitro.40 This was the first clear evidence of any nucleotide binding or kinase activity of a member of the Tribbles homologues family, and suggested a unique catalytic mechanism. Trb2’s proposed Mg\(^{2+}\)-independence is unusual, but not entirely without precedent. The one confirmed exception is the CASK-CaM kinase, an active pseudokinase that is actually inhibited by Mg\(^{2+}\) and employs a residue-only ATP coordination
mechanism. A similar rationale for Trb2’s putative Mg$^{2+}$ independence has not yet been proposed in the literature, but CASK does illustrate that kinase activity is possible without a divalent cation cofactor.

Trb2’s reported kinase activity is unusual given its similarity to Trb1. Trb1 is Trb2’s closest homolog, and their PKD’s share 71% sequence identity. They also share overlapping functional profiles, both inhibiting similar MAPK-kinases and facilitating C/EBPα degradation. As discussed above, the Trb1 crystal structure showed several structural features conserved between the three Tribbles homologues that result in a degraded catalytic cleft incapable of binding ATP. If Trb2 is truly active, this suggests a structural difference permitting nucleotide binding that is not apparent through standard kinase sequence annotation.

There is currently no established functional rationale for Trb2 kinase activity. In some cases, protein phosphorylation activity would actually be contraindicative to known regulatory functions. If Trb2 inhibits Akt as a decoy kinase by binding its activation loop in the catalytic cleft, phosphorylation activity at that site would have an effect opposite to the inhibition of Akt phosphorylation and activity observed in vivo. Additionally, mutating the Trb2 catalytic loop to more closely resemble an active protein kinase eliminated C/EBPα binding and COP1 ubiquitination. Although Trb2 may possess autophosphorylation activity, the current functional evidence does not point towards phosphorylation of protein substrates other than itself. Trb2 autophosphorylation may be autoinhibitory, contributing to PEST-dependent turnover, or causing conformational changes that alter binding affinities. The discovery that Trb2 binds ATP has functional implications independent of any kinase activity. ATP binding could cause conformational changes affecting Trb2 affinity in a manner similar to HER3 or other nucleotide-binding pseudokinases. Further structural and enzymatic characterization of Trb2 and its
interactions with ATP are necessary to understanding the mechanism for its putative kinase function, and *in vivo* experiments will be necessary to determine its biological significance.

### 1.3 Project Overview

The goal of this project was to further investigate and characterize the Mg\(^{2+}\)-independent kinase activity of Trb2. Active pseudokinases are alluring subjects, as they employ uncommon or even outright novel catalytic mechanisms. It is also uncommon for two proteins to be as similar with regards to sequence, phylogeny and function as Trb1 and Trb2 to have such a pronounced difference in relative catalytic activity. The specific aims of this project were to:

1) Develop a method for the high-yield purification of Trb2.

2) Further investigate Mg\(^{2+}\)-independent ATP binding and kinase activity of Trb2.

3) Determine a 3D structure of Trb2 using X-ray crystallography.

After screening >150 expression and purification conditions, a method capable of producing high yields of Trb2 (>10 mg protein per L liquid culture) at >95% purity was developed. This construct was used for biophysical characterization, enzymatic assays, and crystallization screening. Analytical ultracentrifugation showed that Trb2 forms a homomultimer in solution, and we discuss how this could affect the variable signaling potency of the Tribbles homologues. Trb2 was also shown to lack ATP binding or kinase activity, illustrating the challenges inherent to confirming the activity of putatively active enzymes *in vitro*. Crystallization screening has produced several promising leads that could eventually yield diffractable crystals with further optimization and expansion.
Chapter 2
Materials and Methods

2.1 Construct Design and Cloning

All constructs of Trb1, Trb2 and Trb3 were amplified from full-length human cDNA obtained from Origene Technologies (Rockville, MD) (Accession #’s NM_025195, NM_021643, NM_021158). Kinase-domain constructs were optimized for crystallization propensity and minimal disorder using the XtalPred prediction server, which estimates the likelihood of crystallization based on solved structures in the Protein Data Bank. All primers were obtained from Eurofins MWG Operon (Huntsville, AL) and engineered to incorporate BamH1 and Xho1 restriction sites at the 5’ and 3’ ends of the final PCR products. Constructs were amplified from the appropriate cDNA using DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) and subsequently purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific).

PCR products of the target truncations were inserted into expression vectors including pET16b (Novagen, Madison, WI) and a modified pET16b featuring an N-terminal polyHis-MBP-TEV fusion tag. All vectors used also have an ampicillin resistance selection gene, so all E. coli cultures were grown in the presence of 100 ug/L ampicillin. PCR products and vectors were co-digested by BamH1 and Xho1 for one hour at 37 °C, and purified again using the GeneJet Gel Extraction Kit. Vector and PCR product were then ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA) for 25 minutes at 25 °C. Chemically competent TOP10 E. coli cells (Thermo Fisher Scientific) were transformed with the ligation mixture via heat shock, and then plated to agar plates containing Luria-Bertani (LB) broth.
Colonies were screened for successful ligation of the gene into the target expression vector. Five colonies per transformation were used to inoculate 5 mL of LB broth and grown overnight at 37 °C. Plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). To confirm the presence of the insert, subsamples of each plasmid purification were digested with BamH1 and Xho1 for one hour at 37 °C and then electrophoretically separated on a 1% agarose gel containing RedSafe. Fragments were visualized under UV light. Clones positive for insertion were sent to Robarts Research Institute (London, ON) for sequence determination. Finalized constructs used for subsequent expression, purification and downstream experiments are summarized in Figure 2.1.

**Figure 2.1 Linear architecture of human Trb2 constructs**

Linear schematic of finalized Trb2 constructs to be expressed in *E. coli* and purified. Numbers indicate amino acid position relative to the full-length Trb2 sequence.

### 2.2 Expression and Purification of Trb Homologue Proteins

All Tribbles homologues proteins and truncations used were recombinantly expressed in *E. coli* cells. Chemically competent BL21 (DE3) pLysS cells were transformed with T7
expression vectors containing the Trb homologue clones. 50 mL LB starter cultures were inoculated and grown overnight. Scaled-up liquid cultures were inoculated with 12.5 mL of starter culture per litre media and grown at 37 °C with shaking at 220 rpm to an OD_{600} of 0.6-0.8. Cultures were then transferred to a cold room at 4 °C for 1 hour to prevent overgrowth. T7-dependent protein expression was induced by the addition of 0.5 mM IPTG (Bioshop, Burlington, ON), and the cultures were incubated further at 18 °C for 22 hours. Cells were harvested via centrifugation and resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 20 mM Immidazole, 10% glycerol). Cell suspensions were then frozen at -20 °C overnight.

The expression cultures were lysed and the polyhistidine-tagged proteins were purified using immobilized metal affinity chromatography (IMAC). Frozen pellets were thawed for 10 minutes in a room temperature water bath and then incubated on ice with 1 mg/mL lysozyme for 1 hour. Lysate was then sonicated on ice at 30% amplitude with 10-second pulses interspersed with 45-second cooling periods for 3 minutes. The lysate was centrifuged for 30 minutes at 3.0x10^4 x g to remove insoluble components. The supernatant was incubated with 5 mL Ni^{2+}-NTA chelating resin (G-Biosciences, St. Louis, MO) at 4°C for one hour. The protein-bound resin was then washed with 100 mL of lysis buffer plus 20 mM imidazole to remove contaminants. The remaining proteins were eluted from the resin by an imidazole gradient (50 mM, 100 mM, 150 mM, 200 mM, 300 mM). Fractions were selected for content and purity by SDS-PAGE, and combined for further purification.

Protein extracts obtained using IMAC were transferred to storage buffer and further purified. Selected elution fractions were pooled and dialyzed into Trb buffer (20 mM Bicine, 100 mM NaCl, 1 mM DTT, 10% glycerol, pH 9.0) overnight at 4 °C. All assays were performed in Trb Buffer unless otherwise stated. 50 units of TEV protease were included for constructs
expressed from the modified MBP-fusion pET16b vector. The TEV-cleaved protein extracts were incubated with Ni\textsuperscript{2+}-NTA resin as above to remove cleaved MBP and uncleaved MBP-fused protein, and the flow-through containing the cleaved protein was collected. All extracts were concentrated to 1-3 mL using a 10k Amicon centrifugal filter and loaded onto a HiLoad 16/60 Superdex 200 (GE Healthcare) size exclusion column via FPLC (fast protein liquid chromatography). The sample was eluted at 1 mL/minute in 1.5 mL fractions. Fractions were selected based on UV absorption at 280 nm (A\textsubscript{280}) and checked for final purity with SDS-PAGE. Appropriate fractions were pooled and concentrated in another 10 kDa centrifugal filter to 10 mg/mL. Protein concentration was measured based on the absorption at 280 nm and the predicted extinction coefficient (28015 M\textsuperscript{-1}cm\textsuperscript{-1}).

2.3 Analysis of Protein Folding and Ligand Binding via Circular Dichroism

2.3.1 Secondary Structure Deconvolution

The 53-343 Trb2 truncation was analyzed using a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK) to determine secondary structure and assess the quality of protein folding. 30 μM protein in 50% Trb buffer (10 mM Bicine, 50 mM NaCl, 0.5 mM DTT, 5% glycerol, pH 9.0) was loaded into a 0.1 mm cuvette and scanned between 190 and 260 nm in 1 nm increments. The final spectra were averaged over eight scans. Temperature-dependence of CD spectra was assayed in 5 °C increments from 20 to 55 °C. Spectra were deconvoluted using OLIS SpectralWorks and the CONTINLL algorithm (On-Line Instrument Systems, Bogart, GA).

2.3.2 Circular Dichroism Melting Curve

CD spectra of 53Trb2 were collected over a temperature gradient to evaluate ligand- and temperature-dependent changes in secondary structure. Spectra were collected as previous, but in the presence and absence of 3 mM ATP. The sample cuvette was heated from 20 to 55 °C, and
measured at 5° increments. Ligands and buffer contributions to the spectra in the absence of protein were also measured and subtracted from the final averaged spectra. Melting temperature (T_M) was estimated in QtiPlot by plotting changes in molar ellipticity at 208, 218 and 222 nm, fitting the curve to a Boltzmann distribution, and determining the inflection point by finding the maximum derivative.42

2.4 Analytical Ultracentrifugation of 53-343 Trb2

The 53-343 aa truncation of Trb2 was submitted to analytical ultracentrifugation (AUC) to determine its oligomerization state in solution. The protein sample was prepared as previous, but without glycerol or DTT in the Trb buffer (20 mM Bicine, 100 mM NaCl, pH 9.0) to minimize the need to correct for solvent contributions or additive precipitation. 0.5 mg/mL of protein was loaded into a centrifuge cell equipped with double sector charcoal-Epon centerpiece and spun on an An60-Ti rotor in an Optima XL-I analytical ultracentrifuge (Beckman Coulter). Sedimentation experiment was carried out at 35000 rpm at 20°C for 12 hours. Concentration distributions were measured at 150-second intervals with Rayleigh interference optics. The sedimentation coefficient distribution was determined using the program SEDFIT.43

2.5 Fluorescent Reporter Assays for 53Trb2 Ligand Binding

2.5.1 MANT-ATP Analogue Binding

Fluorescent ATP analogues were used to test for 53Trb2 nucleotide binding. The emission spectra of MANT (N-Methylanthraniloyl)-conjugated ATP and ADP were measured on a FluoroLog Spectrophotometer (JY Horiba Inc.) in the presence and absence of 30 μM 53Trb2, 5 mM MgCl2 and 2 mM EDTA. Samples were excited at 365 nm and emission was read between 410 and 470 nm. The final spectra were averaged over eight scans.
2.5.2 SYPRO Orange Thermal Shift Assay

A hydrophobic dye binding thermal shift assay was used to analyze ligand-dependent thermostabilization. The fluorescent yield of 10 μM 53-336 Trb2 and 10% v/v 200x SYPRO Orange Fluorescent Dye was measured on FluoroLog Spectrophotometer in 1 cm quartz cuvette. The fluorescent yield of SYPRO orange is sensitive to the hydrophobicity of its chemical environment. SYPRO orange fluorescence in the presence of protein can therefore be used to assay the effects of ligand binding on thermal denaturation by comparing the melting temperature (T_m) of the protein and protein-ligand complex (Figure 2.2). Samples were excited at 490 nm and emission was measured at 575 nm and 604 nm. The temperature of the sample chamber was increased from 20 to 55 °C in 5 °C increments. The cuvette and chamber were allowed to equilibrate for 2 minutes, and then scanned in triplicate. Scanning was repeated in the presence of 1 mM ATP, 2 mM EDTA, and 10 mM MgCl_2. The fluorescent yields were averaged and normalized to the maximum.
**Figure 2.2 General schematic of hydrophobic dye binding thermal shift assay.**

SYPRO Orange dye (D) fluoresces more intensely when bound to hydrophobic protein surfaces. When proteins unfold in response to increased temperature (ΔT), more hydrophobic surfaces become available for binding and fluorescence increases. Proteins bound to ligand (L) are more resistant to thermal denaturation. Therefore, a greater increase in temperature is necessary to obtain maximum dye fluorescence.

**2.6 ATPase Activity Assay**

53Trb2 was tested for nonspecific ATP hydrolysis activity using the PiColorLock™ Gold Phosphate Detection System (Innova Biosciences, Cambridge, UK). The PiColorLock™ system indirectly measures the liberation of inorganic phosphate (P<sub>i</sub>) through the formation of phosphomolybdate-malachite green dye complexes. The dye complex has an absorption maximum of ~635 nm, the strength of which can be measured in comparison to a P<sub>i</sub> standard curve to determine the concentration of P<sub>i</sub>. 15 μM of 53Trb2 was incubated in the presence or absence of AMP/ADP/ATP (20 mM), EDTA (2 mM) and MgCl<sub>2</sub> (10 mM). 100 μL subsamples were collected in duplicate at 0, 1, 2 and 3 hours and flash-frozen in liquid nitrogen to halt hydrolysis. A confirmed ATPase, ADCK3, was assayed in parallel as a positive control in the presence of ATP and MgCl<sub>2</sub>. ATP by itself was also assayed by itself to estimate the background
contribution of autohydrolysis. Time point samples were thawed and immediately mixed with 25 μL of the malachite green dye. The mixture was allowed to develop for 5 minutes before 10 μL of stabilizer solution was added to halt nucleotide autohydrolysis. Samples were covered and left to develop further for 30 minutes at room temperature. Absorbance at 635 nm was measured with a microplate spectrophotometer (Bio-Tek, Winooski, VT) and final P_i concentration was determined in comparison to a sodium phosphate standard curve.

2.7 32P Radioactive Phosphate Incorporation Kinase Assays

MBP-53Trb2 was assayed for magnesium-independent autophosphorylation activity by measuring the total incorporation of radioactive 32P in solution. MBP-53Trb2 was used to compensate for 53Trb2’s limited solubility in a standard kinase reaction buffer meant to duplicate physiological pH and salinity. MBP-53Trb2 was prepared as described previously, but without the addition of TEV or a second IMAC purification step. 2.5 μM MBP-53Trb2 was incubated with 250 μM cold ATP and 200 CPM/pM [γ-32P]-radiolabeled ATP (PerkinElmer, Waltham, MA) in kinase buffer (20 mM Tris-HCl pH 8.0, 1 mM DTT). Kinase reactions were run in duplicate at room temperature, and in parallel with and without 12 mM MgCl\(_2\). 40 μL aliquots were collected at 5-, 35- and 60-minute time points and transferred to 5 cm\(^2\) pieces of P81 ion exchange cellulose chromatography paper (Whatman International Ltd., Maidstone, U.K.). The papers were washed for 15 minutes in 1.4% phosphoric acid twice and then left to air-dry overnight. The papers were added to 5 mL of ScintiVerse Universal LS Mixture (Thermo Fisher Scientific). Radioactivity was measured in CPM using a Beckman LS 9000 scintillation counter (Beckman Coulter, Brea, CA).
To account for the relatively low sensitivity of the above method and the poor stability of the target protein in solution, the $^{32}$P incorporation of 53Trb2 was assayed again via filmless autoradiography. 36 $\mu$M 53-336 Trb2 in saline kinase buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl) was assayed in the presence of 250 $\mu$M ATP, 100 CPM/pmole [$\gamma^{32}$P]- radiolabeled ATP, with 2 mM EDTA and 10 mM MgCl$_2$ in parallel. Bovine serum albumin (BSA) and ACAT, a confirmed autophosphorylating kinase, were prepared in parallel as negative and positive controls, respectively. Time-points containing ~12 $\mu$g of protein (10 $\mu$L) were collected at 0, 15, 30 and 60 minutes and transferred directly into 10 $\mu$L 2X SDS-PAGE loading buffer to halt the reaction. Samples were separated on a 12.5% SDS-PAGE gel and stained with Coomassie blue. A BAS-Ip Multipurpose Standard Storage Phosphor Screen (GE Healthcare) was exposed with the gels for 30 minutes and scanned on a Typhoon FLA 9500 Biomolecular Scanner (GE Healthcare).

### 2.8 Homology Modeling and of 53Trb2

A homology model of 53Trb2 was generated using the SWISS Model web server.$^{44}$ The crystal structure of the Trb1 pseudokinase domain (PDB: 5CEK) was submitted as the template.$^{31}$ The model was visualized in PyMol.$^{45}$

### 2.9 Crystallization Screening and Optimization

53Trb2 and 53-336 Trb2 were tested for conditions that could produce crystals for X-ray diffraction (Appendix B). Protein was automatically dispensed to 96-well sitting drop vapour diffusion plates containing commercially available sparse-matrix and systematic condition screens by a Phoenix Liquid Handling System (Art Robbins Instruments, Sunnyvale, CA). Protein was dispensed in 0.4 $\mu$L drops at a 1:1 ratio to the mother liquor. Screens were repeated.
at protein concentrations 10, 7, 5 and 2 mg/mL. Potential hits were reproduced and expanded on with 4 µL drops on 24-well hanging drop vapour diffusion plates.

Spherulite microseeding was attempted to improve crystal size and morphology. Spherulite crystallinity and protein content were verified based on Izit Dye (Hampton Research) adsorption. Large spherulites (>0.5 mm in diameter) were looped and suspended in 50 µL of the corresponding mother liquor. The suspension was transferred to a Seed Bead™ tube (Hampton Research) and vortexed at 30-second intervals, interspersed with 30-second cooling periods. The seed stock was then serially diluted in mother liquor (10⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵). Spherulite-producing conditions were then reproduced with an additional 0.5 µL of seed stock added per drop.

Crystals were tested for diffraction on a home X-ray beam source. Crystals were looped from the drops, transferred into cryoprotectant (reservoir solution + 20% glycerol), relooped, and immediately mounted under a liquid nitrogen cryo-stream at 100 K (Oxford Cryo-System). Crystals were exposed to X-rays for 10 minutes by a Rigaku MicroMax-007 (Rigaku) rotating copper anode with 1° oscillation per exposure. Diffraction patterns were collected by a Mar345 (Rayonix) imaging plate.
Chapter 3

Results

3.1 Expression and Purification of Trb2

The human Tribbles homologues were amplified from cDNA clones and inserted into expression vectors for expression in *E. coli*. Over 200 combinations of fusion tags, cell lines, solubilizing additives, expression conditions and purification methods were attempted to produce soluble protein at useable yields for crystallography (Appendix A). Trb1, Trb3 and their truncations proved very difficult to produce at essentially every stage of the process: total yield was close to negligible, and what little could be produced would rapidly aggregate and precipitate when subjected to even gentle lysis. Full-length (FL) Trb2 expressed to some extent in BL21 (DE3) cells at 20°C, but the total yield was low.

FL-Trb2 expression was improved marginally by expressing it in BL21(DE3) pLysS *E. coli* cells at a decreased temperature (18°C). The pLysS cell line features an additional plasmid encoding for lysozyme on a T7 promoter, which reduces the basal expression of target genes by via competition for nonspecific transcription factors. Native Trb2 is unlikely to be toxic to *E. coli*, but unfolded Trb2 produced at growth temperature (37°C) may have limited bacterial growth post-induction. The pLysS cells likely prevented the accumulation of thermally denatured Trb2 prior to induction.

Although it was expressing in high amounts, Trb2 solubility was low following lysis and separation of the insoluble components. Multiple approaches for improving soluble yield were attempted, including the addition of solubilizing additives, varying salt concentrations, and applying different lysis methods. Improvements were marginal until we adapted the purification
method from Bailey et al.’s publication reporting the in vitro kinase activity of Trb2.\textsuperscript{40} They developed a storage buffer that improved the solubility and long-term stability of Trb2, (20 mM Bicine pH 9.0, 300 mM NaCl, 1 mM DTT, 10% glycerol), referred to henceforth as “Trb buffer”.

Although Trb2 was much more soluble in this buffer, the high pH and presence of DTT made it poorly-suited to commonly available affinity purification methods, particularly polyhistidine-nickel IMAC. This reduced affinity made initial purification difficult, as high concentrations of eluting reagent could not be used to remove nonspecifically bound contaminants from the affinity resin without also losing a large amount of the tagged Trb2 in the process. This issue was circumvented by performing the initial lysis and IMAC in a Tris-based buffer (0.1 M Tris-HCl pH 8.0, 300 mM NaCl, 10% glycerol) at 4°C, and then dialyzing the eluted fractions into the storage buffer overnight.
Figure 3.1 Size-Exclusion elution profile of FL-Trb2 following TEV cleavage

Full-length human Trb2 was purified via FPLC on a HiLoad Superdex 200 size-exclusion column. Residual His-tagged MBP cleaved by TEV and partially extracted using IMAC partially co-eluted with Trb2, limiting final purity. The majority of particles in the loaded sample were above the maximum size cutoff of the column (200 kDa) and eluted at the void volume (40 mL), indicating that most of the protein was aggregated.

Following IMAC, size-exclusion chromatography was attempted to further improve the purity of full-length Trb2. The UV absorption of the eluted sample showed that over 50% of full-length Trb2 eluted at the column void volume (40 mL), indicating that extensive non-specific aggregation was occurring (Figure 3.1). A >90% pure sample with a yield of 0.5–1.0 mg per litre liquid culture was attainable via this method. While the maximum concentration was less than ideal for initial crystallography screens, physical and enzymatic analyses were possible. The solubility FL-Trb2 was improved somewhat by the addition of an N-terminal polyHis-MBP tag.
connected by a linker containing TEV protease cleavage site. TEV was able to cleave >90% of the fused protein, but separating polyHis-MBP from the purified Trb2 proved difficult. The two separated poorly on size exclusion despite a >10 kDa difference in molecular mass. A second IMAC purification prior to size-exclusion removed most of the polyHis-MBP, but resulted in poor sample yields that made it difficult to obtain sufficient samples appropriate for crystallization screening. The high level of aggregation despite the drastic improvements to Trb2 solubility suggested that the remaining issues may be intrinsic to the protein.

Truncated Trb2 constructs were designed to improve the protein’s stability in solution. Assuming that the terminal domains and the relatively disordered CTR and NTR segments were cause of Trb2’s aggregation, a pseudokinase domain-only truncation was attempted first. Contrary to expectations, the PKD-only construct showed drastically limited yield and stability compared to the full length. A study by Keeshan et al. (2010) made a similar observation, noting that PKD-minimal Trb2 constructs showed poor yield and solubility, even when expressed with a GST solubility tag. They were able to improve their yield by extending their truncation to include the entire CTR domain, and an additional 20 amino acids of the NTR (see Figure 2.1). Our version of this construct (53Trb2) expressed at much higher levels in pLysS cells than the previous constructs and was successfully purified using Ni^{2+}-NTA resin (Figure 3.2).
N-6x His-tagged 53Trb2 (33 kDa) was expressed in BL21 (DE3) pLysS cells (Lys) and the majority of protein remained soluble following lysis and centrifugation (Sup – supernatant). The polyhistidine tag bound to the Ni$^{2+}$-NTA resin, allowing for the removal of contaminating bacterial proteins (FT - flowthrough, Wash 1 and 2). The bound protein was eluted from the resin with an imidazole gradient (Elutions 1-6). Elution fractions were selected for minimal remaining contaminants and high 53Trb2 content, and then pooled for downstream purification and experiments.

Size-exclusion chromatography of 53Trb2 in combination with the Trb storage buffer showed less aggregation than previous preparations (Figure 3.3A). More than 60% of the protein sample eluted around 80 mL, which is consistent with the molecular mass of unaggregated 53Trb2 (33 kDa). In combination with the “Trb Buffer” for storage and stabilization, we were able to obtain high-purity preparations of 53Trb2 at a final yield of 10-15 mg per liter liquid culture using IMAC and size-exclusion.
Figure 3.3 Size-Exclusion purification of 53Trb2

53Trb2 purified using IMAC was further purified via FPLC on a HiLoad S200 size exclusion column. (A) Disperse 53Trb2 eluted from the column between 70 and 90 mL. (B) SDS-PAGE analysis confirmed the identity and purity of the 53Trb2 peak (~33 kDa). Representative fractions from the contaminating peaks (C1 and C2) show bands consistent with 53Trb2 and unidentified bacterial contaminants, possibly in complex with one another.
The size-exclusion profile of 53Trb2 suggested the possibility of multimeric complexes in solution. The 53Trb2 peak was broad, overlapping with a smaller peak eluted around 65 mL. Another small peak that was absent in previous preparations eluted around 50 mL. SDS-PAGE of representative fractions from the two peaks (Figure 3 C1 and C2) indicated the presence of two large contaminating proteins (75 and 72 kDa), and trace amounts of 53Trb2 (Figure 3.3B). The contaminants could be bacterial chaperonins such as DnaK or Hsp70, which can be retained during protein purification in complexes with the target protein if large regions of it are unfolded. The wide elution range of 53Trb2 also suggested the possibility of 53Trb2 oligomers. Additional characterization of 53Trb2’s fold state and oligomerization was necessary to explain these observations.

3.2 Secondary Structure Analysis of 53Trb2

Although it was much more soluble than the full-length and PKD-only constructs, 53Trb2 still showed signs of aggregation on size exclusion. The secondary structure of the purified 53Trb2 was analyzed using CD to confirm that the unaggregated fractions collected remained folded. The Trb storage buffer contains high concentrations of NaCl and bicine, which resulted in a high background and limited the spectrum coherence at lower wavelengths. Diluting the buffer by 50% with the water reduced the background enough to obtain a useable spectrum. The spectra showed that 53Trb2 was primarily α-helical with some β-strand character (Figure 3.4), which is consistent with a folded ePK domain.
Figure 3.4 CD spectra of 53Trb2

The secondary structure of 53Trb2 was analyzed via CD. 30 μM 53Trb2 in 50% Trb Storage Buffer was scanned in a 0.1 mm cuvette at 25 °C. Eight scans taken between 190 and 260 nm in 1 nm increments were averaged to obtain a final spectra applied to secondary structure deconvolution.

Deconvolution of the CD spectra shows that the secondary structure composition of 53Trb2 is largely consistent with the homology model prediction (Table 3.1).

Table 3.1 Deconvolution of the CD 53Trb2 spectra

<table>
<thead>
<tr>
<th>Structural Motif</th>
<th>Homology Model Prediction</th>
<th>CD Deconvolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Alpha Helix</td>
<td>33</td>
<td>35</td>
</tr>
<tr>
<td>% Beta Strand</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>% Turn</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>Coil/Other</td>
<td>24</td>
<td>31</td>
</tr>
</tbody>
</table>
3.3 Analytical Ultracentrifugation of 53Trb2

53Trb2 was submitted to analytical ultracentrifugation to determine its oligomerization state in solution. The Lamm equation fit of the data shows distinct peaks corresponding to unaggregated Trb2. However, it also indicates a mixed population of monomers (53%), dimers (19%), tetramers (10%), and possibly higher-order oligomers (18%) (Figure 3.5B). This confirms that the broad 80 mL peak and smaller accessory peaks containing 53Trb2 observed in the size-exclusion elution profile were reflective of homomultimer formation.
Figure 3.5 Sedimentation distribution of 0.5 mg/mL 53Trb2

53Trb2 was submitted to AUC to determine its oligomerization state in solution. 0.5 mg/mL of purified protein was sedimented at 35000 RPM for 12 hours on an Optima XL-I AUC. (A) Radial distribution of protein concentration was measured via Rayleigh interferomics at 150 second intervals. (B) Sedimentation coefficient distribution calculated using SEDFIT shows a size distribution consistent with multiple 53Trb2 homomultimers in solution.

3.4 Radiolabeled ATP Kinase Assays

Radiometric assays were used to reproduce the Trb2 Mg\(^{2+}\)-independent autophosphorylation activity reported by Bailey et al.\(^{40}\) The protein was incubated in the presence of radiolabeled \(^{32}\)P\(_\gamma\)-ATP and either MgCl\(_2\), or EDTA, which would chelate any
contaminating divalent cations. The incorporation of radioactive phosphate into the protein was then measured using both liquid scintillation counting and filmless autoradiography.

Trb2 autophosphorylation site(s) have not been identified. Therefore, the assay was first attempted with MBP-Trb2, as the full-length protein would include all possible phosphorylation sites. However, MBP-Trb2 precipitated out of solution before the full time course could be completed. The experiment was repeated with MBP-53Trb2 instead, which had previously demonstrated better thermostability.

Liquid scintillation and filmless autoradiography were used to evaluate the incorporation of radioactive $^{32}$P into Trb2. Liquid scintillation allows for precise quantitation and requires minimal sample processing, but is susceptible to high background and cannot discern the exact source of radioactivity in a sample. Filmless autoradiography is more sensitive, and samples can be separated via SDS-PAGE prior to imaging. However, the high sensitivity of autoradiography makes it susceptible to false positives. 100 CPM/pmole hot ATP was used, as higher concentrations increases the probability of false positives arising from contaminating bacterial kinases or non-specific phosphotransfer.
Filmless autoradiography (Figure 3.6A) and liquid scintillation (Figure 3.6B) both showed no evidence of any time-resolved incorporation of radioactive phosphate into Trb2, with or without Mg$^{2+}$. The positive controls, ADCK3 and ACAT, have weak autophosphorylation activity compared to most typical kinases, but still displayed evidence of phosphate incorporation. 53Trb2 in the presence and absence EDTA and MgCl$_2$ were incubated with 100 CPM/pmole radiolabelled $[^{32}\text{P}]$-ATP to test for Mg$^{2+}$-independent autophosphorylation. (A) 10 μL Time points were collected at 0, 15, 30 and 60 minutes and transferred to SDS-PAGE loading buffer to halt the reaction. Samples were separated via SDS-PAGE and stained with Coomassie blue (top). A phosphor storage screen was exposed with the gels for 30 minutes, and then imaged to visualize the incorporation of radiolabeled phosphate incorporation assays for 53Trb2 kinase activity.
incorporation in the conditions assayed. Based on these results, it cannot be confirmed that Trb2 is Mg\textsuperscript{2+}-dependent or -independent autophosphorylating kinase.

3.5 ATPase Activity Assays

The kinase assays conducted using 53Trb2 did not eliminate the possibility that Trb2 autophosphorylates one or more of the first 52 N-terminal residues omitted from the construct used in the radiolabeling assays. This issue can be circumvented by measuring ATPase activity, as the majority of active kinases display nonspecific ATP hydrolysis activity independent of substrate.\textsuperscript{47} Assuming the disordered NTR does not serve a direct catalytic function, the 53Trb2 construct should display ATPase activity if Trb2 is an active kinase.

MBP-Trb2 and MBP-53Trb2 showed no evidence of Pi liberation over time in excess of background ATP hydrolysis (Figure 3.7). Background ATP hydrolysis was high compared to other studies, between 1-5 µM Pi. The high background is likely due the relatively high pH of Trb buffer (pH 9.0), as ATP is more prone to spontaneous hydrolysis in alkaline conditions. The assay was attempted with a more neutral buffer, 20 mM Tris-HCl at pH 7.0, but this caused the protein form opaque precipitates in the resolving solution that made it impossible to accurately measure the absorption of the dye complex. Due to the lack of discernible Pi liberation, we suggest that Trb2 or 53Trb2 does not have any catalytic activity towards ATP \textit{in vitro}.
Figure 3.7 Trb2 ATPase Activity

The ATP hydrolysis activity of MBP-fused full length Trb2 and the 53Trb2 truncation was assayed in the presence and absence of MgCl$_2$ and EDTA using the PiGold Color Lock kit. Neither Trb2 construct showed any evidence of time-resolved Pi liberation. The differences in baseline is likely attributable to variations in Pi background contamination between the separate protein preparations.

3.5.1 Binding of MANT-ATP Fluorescent ATP Analogs

To test that the Trb2 PKD binds ATP, the fluorescent spectra of MANT-conjugated ATP analogs was measured in the presence of 53Trb2. The MANT (N-Methylanthraniloyl) moiety can receive excitation potential from adjacent aromatic groups through FRET. If the conjugated nucleotide binds to a protein, the fluorescent yield of MANT should increase as long as aromatic amino acids (Tyr, Phe, Trp) are nearby. Based on the emission spectra (Figure 3.8), 53Trb2 did not have a large effect on the fluorescent yield of MANT-conjugated ATP. The fluorescent yield of MANT-ATP increased 4% in the presence of only 53Trb2 or 53Trb2 with EDTA, but decreased by the same amount in the presence of 53Trb2 and MgCl$_2$. This is a small change in fluorescent emission compared to confirmed instances of MANT-ATP binding, for which a two
to fivefold increase is expected. At most, this suggests a weak or no interaction between ATP and 53Trb2 that is not influenced by the presence of Mg$^{2+}$.

Figure 3.8 Evaluation of 53Trb2 nucleotide binding via MANT-ATP fluorescence

The fluorescence of a MANT-conjugated ATP analogue was used to evaluate 53Trb2 ATP binding in the presence and absence of 5 mM MgCl$_2$ and 2 mM EDTA. MANT-ATP fluorescent yield decreased slightly in the presence of 30 μM of protein, and increased both with MgCl$_2$ and EDTA. Shifts of this magnitude are insufficient to confirm MANT-ATP binding to 53Trb2 in the conditions assayed.

3.6 Thermal Shift Assays

Two types of thermal shift assays (TSA), differential scanning fluorimetry (DSF) and a CD melting curve were applied to assess whether or not ATP binds Trb2, and how that binding is affected by the presence of Mg$^{2+}$. Both methods can be used to determine ligand-dependent shifts in a protein’s melting temperature ($T_M$). $T_M$ is defined as the temperature at which the population of folded ($P_f$) and unfolded ($P_U$) are equal. Assuming that a binding interaction between protein and ligand improves the thermodynamic favorability of the folded state, changes in $T_M$ can be used to indirectly analyze protein-ligand interactions.
3.6.1 CD Melting Curve

Circular dichroism can be used to measure changes in the secondary structure and global fold state of a protein in response to ligands, denaturants and temperature. Changes in the ellipticity of 53Trb2 in response to a temperature gradient was measured with and without ATP (3 mM) to assess ligand-dependent thermostability. Signals at 208, 218 and 222 nm were monitored to estimate the melting curves, as those wavelengths are generally reflective of α-helical and β-sheet content (Figure 3.9). The melting temperature ($T_M$) of 53Trb2 is defined as the temperature at which the populations of folded ($P_f$) and unfolded ($P_U$) are equal, and is indicative of the overall thermodynamic favorability of the folded state. The $T_M$ at each wavelength was calculated by fitting the ellipticity signal to a sigmoidal curve using the Boltzmann function, and then calculating the maximum derivative (Table 3.2).
Figure 3.9 52Trb2 ATP-dependent CD melting curves

Molar ellipticity versus temperature of 30 μM 53Trb2 with and without 3 mM ATP at (A) 208 nm, (B) 218 nm and (C) 222 nm. Curves represent iteratively-fitted Boltzmann distributions plotted in QtiPlot ($R^2 \geq 0.93$) and vertical red lines indicate melting temperature ($T_M$). Calculated $T_M$ values are summarized in Table 1.2.
The absolute $T_M$ varied between wavelengths, possibly representing independent unfolding of separate secondary structure elements occurring at different temperatures. All three wavelengths indicate an average $\Delta T_M$ of +4.8 °C in the presence of 3 mM ATP. Based on 53Trb2’s improved thermostability, this shows that 53Trb2 appears to bind ATP at millimolar concentrations.

### 3.6.2 Differential Scanning Fluorimetry

DSF is similar in principle to differential scanning calorimetry (DSC), in that it can measure changes in a protein’s fold state over a temperature gradient. Instead of directly measuring the heat of unfolding, DSF measures the binding of a fluorescent dye to the protein. The dye, SYPRO Orange, fluoresces more when in a hydrophobic environment. As proteins unfold in response to increasing temperature, the fluorescent yield of SYPRO increases as more hydrophobic surfaces become accessible.

DSF of 53Trb2 produced clear sigmoidal melting curves fitted using the Boltzmann function ($R^2 > 0.93$), indicating an absolute $T_M$ in the range of 40 °C (Figure 3.10), which is consistent with previous studies.\(^4^0\) 53Trb2 in the presence of EDTA, MgCl\(_2\) and ATP did not show a substantial shift in the $T_M$ relative to 53Trb2 in the absence of additives (Table 3.3). A $\Delta T_M$ of at least +/- 2 °C is considered the minimum shift to reveal a protein-ligand interaction.

### Table 3.2 53Trb2 $T_m$ value calculated base on CD melting curve

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>+ATP ($^\circ$C)</th>
<th>-ATP ($^\circ$C)</th>
<th>$\Delta T_m$ ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>208</td>
<td>48.0</td>
<td>42.5</td>
<td>5.5</td>
</tr>
<tr>
<td>218</td>
<td>39.7</td>
<td>34.7</td>
<td>5.0</td>
</tr>
<tr>
<td>222</td>
<td>39.9</td>
<td>36.1</td>
<td>3.8</td>
</tr>
</tbody>
</table>
with DSF. The $T_M$ of 53Trb2 appeared to decrease in the presence of ATP with either EDTA or MgCl$_2$, which would indicate a denaturing effect.

**Figure 3.10 Ligand-dependent DSF of 53Trb2**

Temperature-dependent denaturation of 53Trb2 in the presence and absence of 1 mM ATP, 2 mM EDTA and 10 mM MgCl$_2$ measured via SYPRO Orange fluorescence. Temperature was increased from 20 to 60°C in 5°C increments. Data was plotted and fit to Boltzmann distributions in QtiPlot. Calculated $T_M$ values are summarized in Table 1.3.
Table 3.3 Ligand-dependent $T_M$ of 53Trb2 determined via DSF

<table>
<thead>
<tr>
<th>Additive</th>
<th>$T_{M}$ (°C)</th>
<th>$\Delta T_{M}$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+EDTA</td>
<td>37.8</td>
<td>-1.7</td>
</tr>
<tr>
<td>+MgCl$_2$+ATP</td>
<td>38.1</td>
<td>-1.4</td>
</tr>
<tr>
<td>*</td>
<td>39.5</td>
<td>---</td>
</tr>
<tr>
<td>*</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td>*</td>
<td>39.6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The two TSA experiments present conflicting results as to the ATP affinity of Trb2. The CD melting curve shows weak ATP-dependent thermostabilization of 53Trb2 secondary structure, whereas DSF does not show a large enough change to confirm any sort of ligand interaction. Further experimentation is necessary to fully confirm or refute Trb2-ATP binding, although such weak binding, if real at all, is unlikely to have any significant functional implication.

3.7 Crystallization of Trb2

3.7.1 Spherulite Microseeding

53Trb2 with 5 mM ATP formed slightly angular orbs in multiple conditions containing magnesium formate or sodium malonate, and at pHs between 6.0 and 8.0 (Figure 3.11A). The orbs behaved as solids when prodded with a touching tool, suggesting some degree of crystalline organization as opposed to simple phase separation. Izit dye (Hampton Research) was added to a well containing one of the pseudo-crystals to confirm they were crystalline protein spherulites, as opposed to salt or liquid-liquid phase separation. Izit dye, a solution of tetramethylthionine chloride, is a dark blue dye that can penetrate the relatively large solvent channels of macromolecular crystals, but not the tightly packed lattice of small-molecule crystals. If a crystal is indeed protein, it will absorb the dye and take on a dark blue colour. The spherulites tested
absorbed Izit dye, confirming that it is crystalline protein (Figure 3.11B). Spherulites are not viable for X-ray diffraction, but are good sources of seed stocks for microseed screening. Additive screens, decreased temperatures and microseeding using the spherulites as stocks so far have only yielded more spherulites.

![Image of spherulites](image)

**Figure 3.11 53Trb2 spherulites obtained through crystallographic screening**

(A) Representative 53Trb2 spherulites. 7 mg/mL 53Trb2 with 5 mM ATP after 1 week of vapour diffusion in 1:1 protein solution to reservoir liquid (1M Tris-HCl pH 8.0, 0.3 M Mg Formate).

(B) A 53Trb2 spherulite before (top) and after 20 hours (bottom) following the addition of 0.5 μL Izit dye. The intense absorption indicates crystalline protein.

3.7.2 53-336 Trb2 Crystallization Screening

53Trb2 was prone to denaturation in crystallization experiments, aggregating in opaque, amorphous precipitate in >50% of the conditions in sparse matrix screening. Reducing the initial protein concentration and varying the drop ratios did not significantly reduce aggregation, suggesting a problem intrinsic to the physiochemical properties of the protein. The seven most C-terminal residues (NLFPFFN) of Trb2 are relatively hydrophobic, and the equivalent region was not found in the Trb1 crystal structure.31 This suggests that the region negatively impacts
solubility, and is too disordered to contribute to crystal packing. A truncated construct lacking those residues (53-336 Trb2) was produced in our attempt to improve solubility and crystallization.

**Figure 3.12 53-336 Trb2 crystals obtained by initial screening and subsequent expansion**

A single crystal of 53-336 Trb2 was produced using the Qiagen Classics I Suite, dispensed via robot to sitting-drop vapour diffusion plates (left). The condition was expanded on with manually-prepared hanging-drop vapour diffusion, producing multiple tetrahedral crystals (right). The largest crystals are approximately 1x1 mm.
Initial sparse matrix seeding of 53-336 Trb2 resulted in new crystal hits in previously unproductive conditions. The most promising hit was found in condition #58 of the Qiagen Classics Suite I (0.1 M Tris-HCl pH 8.5, 10 mM NiCl₂, 1.0 M LiSO₄) with 5 mg/mL protein in a 0.8 µL 1:1 drop at room temperature (Figure 3.12). A single crystal formed after one week, whereas 53Trb2 aggregated within 24 hours in the same condition. The crystal was likely protein, as it fluoresced weakly under UV light. A 24-well reproduction and expansion was prepared with varying concentrations of NiCl₂ and LiSO₄ using 5 and 2 mg/mL. 53-336 Trb2 in 4 µL drops. Large tetrahedral crystals formed within two days in an adjacent condition (0.1 M Tris-HCl pH 8.5, 10 mM NiCl₂, 1.0 M LiSO₄) at both protein concentrations. The new crystals also fluoresced weakly under UV. The three largest crystals were exposed to X-rays on our home source, but did not show any evidence of diffraction. It can be concluded that the 53-336 Trb2 crystals derived from this condition are not salt and most like protein which remain intact in 20% glycerol cryoprotectant, but will require further optimization and testing to obtain diffraction.

3.8 Trb2 Homology Model

The recently published structure of Trb1 was used to generate a homology model of 53Trb2 on the SWISS Model web server. The NTR was omitted due to its high flexibility and sequence variability between the target and template. The model covers 82% of the submitted sequence and has a QMEAN score of 0.84, indicating moderate quality compared to experimentally derived structures. While insufficient to draw any meaningful conclusions about differences in side chain orientations that could explain the reported variations in Trb enzymatic activity, it can be concluded based on the quality of the homology model that Trb1
and Trb2 have very similar tertiary structures. This is unsurprising, given that the sequence identity between the two is 71%.
Chapter 4

Discussion

4.1 Structural Insights from Differential Stability of Trb2

Obtaining a useable preparation of Trb2 proved difficult, requiring extensive experimentation and optimization involving nearly 200 different conditions. Even when scaled up to the necessary concentrations, full-length Trb2’s poor thermostability resulted in inconsistent results from preliminary experiments. Only the combination of the 53Trb2 construct and the Trb storage buffer were sufficient to produce a purified sample that could be reliably applied to assays and crystallographic screening.

The challenges experienced expressing and purifying the full-length and PKD-minimal constructs of Trb2 indicate that the NTR and C1BD strongly influence the stability of the protein. Full-length Trb2 expressed to a reasonable degree in E. coli, but was prone to nonspecific aggregation, and would quickly precipitate out of solution at higher concentrations and temperatures. The PKD-only construct was tried next, under the assumption that most eukaryotic kinase domains are modular, and therefore not dependent on flanking domains to maintain tertiary structure.51 The PKD-only construct showed poor expression under the same conditions, which is consistent with similar observations made by Keeshan et al. (2010).19 They also found that inclusion of residues 53-72, immediately preceding the PKD, and the entire CTR, restored expression to levels comparable to the full-length construct. In combination with the buffer system developed by Bailey et al. (2015), this construct proved much less prone to aggregation when purified via size exclusion, and remained soluble at room temperature for long
periods of time.\textsuperscript{40} Overall, this indicates that the Trb2 NTR confers net instability to the protein, and the CTR stabilizes the PKD.

The relative instability of the full-length Trb2 construct conferred by the NTR is likely caused by its high concentration of PEST sequences.\textsuperscript{52} PEST sequences are primarily considered protease or ubiquitin ligase recognition sequences, so an exogenously expressed and purified PEST protein like Trb2 would not be expected to experience proteolytic/proteasomal degradation \textit{in vitro}, in the absence of the appropriate endogenous enzymes. However, PEST sequences have been shown to increase protein turnover \textit{in vivo} even when recognition elements specific to their degradation mechanisms have been removed. This suggests that while specific inhibitory pathways have evolved around PEST sequences, their primary effect on protein lifetime is to contribute intrinsic disorder to the protein. This would explain full-length Trb2’s propensity for precipitation and aggregation in the absence of proteolysis.

In contrast to the NTR, inclusion of the Trb2 CTR drastically improved protein yield and stability. This suggests a stabilizing domain-domain interaction between the CTR and the central PKD, which is a common feature among ePKs. The Trb1 crystal structure shows a close interaction between the C1BD and a hydrophobic patch opposite the catalytic cleft, which was confirmed in solution via SAXS.\textsuperscript{31} Given the sequence similarities between Trb1 and Trb2, and the PKD/CTR codependence of Trb2 scaffolding complexes, it is likely that the Trb2 PKD and C1BD interact similarly. There was no appreciable change in stability or CD spectra when residues 337 to 343 were omitted, which is consistent with the previously determined C1BD domain boundaries. It is therefore possible that the C1BD enhances Trb2 solubility by concealing the hydrophobic patch on the PKD. Together, these observations support the NTR-
dependent lifetime regulation proposed based on bioinformatic prediction, and strongly indicates
that the Trb2 C1BD interacts intramolecularly with the PKD like Trb1.27

4.2 Functional Implications of Trb2 Oligomerization

Upon observing that the unaggregated population of 53Trb2 behaved liked a multimer
when submitted to size-exclusion chromatography, AUC was applied to analyze the
oligomerization state of the protein. The best-fit of the Lamm equation shows that almost half of
the unaggregated population of 53Trb2 is self-associated into dimer, tetramers, and high-order
multimers. Although it has been suggested previously that Trb2 is capable of
hyperoligimerization, this is the first direct experimental evidence that it self-associates in an
ordered manner. This is not unexpected, as more than a third of the human proteome is predicted
to form some kind of homomultimer.53 Trb1, however, was shown via SEC-MALS and SAXS to
be exclusively monomeric in solution.31 It is unusual for two proteins with such similar
sequences (71%) to have such divergent quaternary structures. Variability in the C1BD
intramolecular sequestration surface could possibly explain this discrepancy. The Trb1 crystal
structure shows the valine of the conserved VPE (358-360) sits in a hydrophobic groove created
by Ile135 and Ile146, and the glutamate forms polar interactions with Tyr97 and Arg102. Despite
the flexibility necessary for this interaction between distal termini, all in-solution evidence
indicates that the C1BD only binds the Trb1 PKD in cis. The Trb2 C1BD sequence is similar,
featuring an analogous VPD (329-331) motif, but there are noteworthy differences in the binding
surface on the N-terminal lobe. The Trb2 homology structure shows that the hydrophobic groove
is still present, but consists of a phenylalanine (Phe105) and an isoleucine (Ile116). Asp331 is
oriented away from the polar-interaction tyrosine, and there is no nearby equivalent to Trb1
Arg102 to aid in binding. The Trb2 N-lobe contacts are still sufficient to allow for C1BD-PKD
binding, but the decreased coordination of the C1BD aspartate could result in a more transient interaction. If the Trb2 C1BD is more mobile, it could have a higher probability of encountering and binding another Trb2 molecule in trans.

Figure 4.1 Structural alignment of Trb1 and Trb2 C1BD intramolecular binding
Alignment of the Trb1 crystal structure (Green) and the Trb2 homology structure (Purple) shows the differences in binding conformation between the C1BD and the N-terminal lobe of the PKD. Glu369 in the Trb1 C1BD forms polar interactions with Tyr127 and Arg132 of the αC helix. The Trb2 structure shows the equivalent Asp331 to be twisted away from the helix surface, restricting polar contacts. The C1BD valines of Trb1 and Trb2 both sit in a hydrophobic groove formed by Ile135 and Ile146, Phe105 and Phe116, respectively.

Conserved differences in multimerization could explain in part the differences in Trb1 and Trb2 regulatory function. The Tribbles Homologues all have closely overlapping functional profiles, but their regulatory effects on the same pathways are variable. Trb2 has been shown to inhibit the phosphorylation of Akt in vivo less intensely than Trb3, and some variability in C/EBPa degradation by Trb1 and Trb2 has been observed.34,39 The formation of a multimer can enhance a protein’s activity by increasing the local concentration of binding sites, or inhibit it by
concealing them. Differences in multimerization potential could therefore be the structural basis for differences in signaling potency between the Tribbles Homologues. Trb2 multimerization could limit the availability of Akt interaction sites relative to Trb3, possibly competitively binding the Trb2 activation loop in \emph{trans}. Similarly, Trb2 multimers could form by the C1BD and PKD interacting \emph{in trans}, which could affect the recruitment of components of the C/EBPα ubiquitination complex.

Trb2 multimers may also play a role in turnover regulation. The PEST sequences in the Tribbles homologues NTRs are predicted to limit the intracellular lifetime of the proteins, which is a common regulatory mechanism for regulators of cellular proliferation. Trb2 multimerization could shield the PEST sequences from proteolytic factors, increasing the long-term stability of the protein \emph{in vivo}. If Trb2 is stabilized by multimerization, it then follows that Trb1 has a shorter lifetime and its signaling function operates over a shorter timeframe.

Trb2 dimerization adds an additional dimension to the possibility of a Trb1/Trb2 heterodimer. An ongoing high-throughput affinity MS study has shown that Trb1 and Trb2 interact with each other to some extent. To our knowledge, this interaction has not been confirmed elsewhere in the current literature, and the effects of Trb1/Trb2 double knockdown or overexpression \emph{in vivo} have not been explored. Although Trb1 proved difficult to produce for the purposes of X-ray crystallography, the amounts we have obtained are sufficient to confirm an interaction between it and Trb2 using affinity pulldown chromatography. If this proves successful, AUC and analytical SEC could then be used to determine the stoichiometry of the hypothetical heterocomplex.

Further investigation of the structural basis of Trb2 multimerization is necessary to understand the possible functional consequences. The reason for Trb1’s contrasting dispersity
remains puzzling given their similarity. A truncation-based strategy to identify the regions involved in self-association is not feasible given the instability of most minimal Trb2 constructs, making it difficult to explain without an experimentally-derived structure of Trb2. If Trb3’s oligomerization state can be determined, it could be possible to identify relevant sequence elements through alignment.

4.3 Trb2 is Catalytically Inactive

Contrary to the findings of Bailey et al. (2015), we did not find any evidence of Mg\textsuperscript{2+} independent autophosphorylation by 53Trb2. Multiple experimental approaches showed no evidence of incorporation of radioactive \textsuperscript{32}P with or without Mg\textsuperscript{2+}, indicating a total lack of kinase activity. The simplest explanation is that the 53Trb2 truncation lacks crucial residues present in the full-length protein used previously. MBP-fused FL-Trb2 rapidly precipitated under assay conditions despite multiple attempts to improve solubility, so the lack of activity observed cannot be considered representative of the native protein. Currently, no specific Trb2 phosphorylation sites have been identified. The NetPhos prediction server identifies multiple serine and threonine residues as probable phosphorylation sites, several of which are in the 52 NTR residues omitted from the 53Trb2 construct.\textsuperscript{55} If the only targets of Trb2 autophosphorylation are in the NTR, no incorporation of radiolabeled phosphate by the 53Trb2 truncation could occur. If this were the case, it would provide some clues as to the function of Trb2 autophosphorylation based on predictive sequence annotation. The Trb2 NTR is rich in PEST sequences, and PEST-mediated proteolysis is often promoted by phosphorylation at one or more PEST ser/thr residue.\textsuperscript{24,56} However, the absence of appropriate phosphorylation sites does not explain the complete lack of Trb2 ATPase activity.
The 53Trb2 construct did not display any nonspecific ATP hydrolysis activity, which is unexpected if it has catalytic activity. Most active kinases catalyze the nonspecific hydrolysis of ATP in the absence of substrate to some extent and the 53Trb2 truncation should still possess all necessary catalytic residues. These conflicting findings suggest that the activity observed by Bailey et al. appears to be a false positive. The radiolabeled phosphate incorporation method used is highly susceptible to nonspecific phosphorylation by contaminating host organism kinases. The risk of such an error is especially high when saturating concentrations of $[^{32}\text{P}]-\gamma$-ATP (>2000 CPM/pmole ATP) are used in conjunction with a method as sensitive as autoradiography, as was the case in the previous study. If the phosphate incorporation of Trb2 is as high as reported (0.01 mol incorporated per mole protein), much lower quantities of radionucleotide should have been sufficient to detect phosphorylation with enough development time. Less radiolabeled ATP (100 CPM/pmole ATP) was used in this study to minimize the rate of false positives. At the incorporation rate reported previously and the quantity of protein used, this would result in a signal on the order of ~400 CPM per sample, which is within the dynamic range of the phosphor screen. Whether or not the observed loss in signal relative to the previous study is the result of bacterial contamination could be confirmed by repeating the incubation in the presence of E. coli cell lysate.

If Trb2 is catalytically inactive, our findings conflict with the previous mutational analysis of Trb2 autophosphorylation. Lys90 in Trb2 is equivalent to Lys72 of PKA, and is crucial to ATP binding in active kinases. A 50% reduction in autophosphorylation by the Lys90Met mutant was reported. Substitution of Lys72 or its equivalent with a non-basic residue is the standard method for the mutational confirmation of kinase activity and nucleotide binding. Considering that Trb2 is predicted to have mitigated kinase activity due to its lack of
a DFG loop, it is unexpected that a mutation that would completely mitigate activity in a canonical kinase only partially affects Trb2. It is possible that the observed loss in activity by the Trb2 Lys90 mutant reflects a change in key structural motifs, rather than a loss of catalytic coordination. Lys72 substitution is used to confirm kinase activity under the assumption that changing the residue will not otherwise alter the structure or stability of the affected domain. This may not be the case with Trb2, as the homology structure suggests an alternative structural role for the equivalent Lys90 residue. In many canonical ePKs, the Lys72 forms a salt-bridge with Glu91 to further stabilize the active conformation in addition to binding ATP. Trb2 lacks an equivalent glutamate residue, but the Trb2 homology model predicts an electrostatic interaction (~2.7 Å) between Lys90 and the backbone hydroxyl of Glu196 in the SLE motif. Mutating Trb2’s catalytic motifs to more closely resemble an active serine kinase sequences has been shown to inhibit Trb2 binding to regulatory effectors in vivo, and Bailey et al. also observed that mutating the SLE loop to a canonical DFG sequence decreased Trb2’s stability. This suggests that the SLE motif contributes to the stable conformation of the Trb2 PKD, and that Lys90 could facilitate this in part through its interaction with Leu196. It is therefore possible that mutations disrupting this interaction could result in changes to the overall conformation of Trb2, limiting the availability of nonspecific phosphorylation sites. If this is the case, then Lys90Met mutation’s loss of 32P incorporation may reflect a Lys90-dependent conformational shift, rather than disruption of an active catalytic motif (Figure 4.2). This unusual conformation of the catalytic lysine not only suggests an alternative structural role, but that stabilization by the invariant SLE motif may prevent it from participating in ATP binding altogether.
If the function of Lys90 is inessential to kinase activity, it may be tempting to suggest that Trb2 employs an alternative ATP-binding mechanism. WNK1, as previously mentioned, is an active pseudokinase that substitutes an alternate lysine residue (Lys233) into the nucleotide binding pocket.\(^8\) Trb2 does have an arginine at the equivalent residue that could serve a similar function (Arg77). However, the homology model shows that it is oriented away from the binding site, and is held in place there by an electrostatic interaction with a nearby glutamate (Glu86). A WNK1-like alternative mechanism is therefore unlikely, as it would be difficult for Arg77 to enter the catalytic cleft to coordinate ATP. It is also worth noting that unlike WNK1, Trb2 lacks a flexible P-loop or an intact DFG loop that could compensate in part for the alternatively coordinated Lys90.

**Figure 4.2 Predicted electrostatic interaction between Trb2 Lys90 and the conserved SLE motif**

The Trb2 homology model suggests that Lys90 (green) - equivalent to the critical catalytic Lys72 in PKA - forms a putative hydrogen bond with backboned carbonyl of Leu196 in the SLE motif (yellow).
Figure 4.3 Comparison of alternative ATP binding residues in Trb2 and WNK1

The WNK1 kinase (right) lacks the canonical ATP-binding lysine, but is still active as the Lys233 in subdomain I is able to enter the catalytic cleft in the active conformation. Trb2 has an arginine at the same position (Arg77) that could perform the same function. This is unlikely, the Trb2 homology model (left) shows that a nearby glutamate (Glu86) forms electrostatic contacts with the residue that would keep it aligned away from the catalytic cleft.

Another questionable element of the autophosphorylation reported by Bailey et al. is the decrease in activity observed when Trb2 and its mutants were expressed in eukaryotic Sf9 insect cells. This is unexpected if Trb2 is an active kinase, as it would be more likely to receive activating post-translational modifications when produced in a eukaryotic environment. The Tribbles homologues are not present in invertebrates, but Tribbles, their evolutionary precursor, is conserved throughout the insect taxa. Trb2 has the sequence features of an “RD” kinase, which implies the presence of one or more activating phosphorylation sites. It then follows that if Trb2 is an active kinase, it would be more likely to encounter activating protein kinase factors when expressed in Sf9 insect cells than in E. coli. This indicates that if human Trb2 does have catalytic activity in vivo, it is much lower than the 0.1% incorporation estimated by Bailey et al.,
and increases the probability that the $^{32}$P incorporation observed was an artifact arising from the protein purification process.

Our attempts to confirm the Mg$^{2+}$-independent activity of Trb2 were ultimately negative, indicating that the previously reported activity reported by Bailey et al. may be a false positive. Analysis of the Trb2 homology structure shows that the mutagenic analysis of Trb2 did not adequately control for alternative structural roles of Lys90, and the experimental conditions applied suggest an unacceptably high possibility of phosphorylation by contaminating bacterial kinases.

**4.4 Trb2-ATP Binding**

Trb2 is likely inactive due to an inability (or very weak ability) to bind ATP. However, binding was demonstrated previously by a TSA approach that would not be susceptible to the same sources of error as radiolabeled phosphate incorporation. Additionally, it is possible that Trb2 can still bind ATP through an alternative mechanism that does not allow it to adopt a catalytically active conformation, as is the case with several other pseudokinases, such as HER3. It is therefore necessary to address Trb2-ATP binding as a distinct phenomenon.

In contrast once again to Bailey et al.’s findings, ATP binding was not observable across multiple experimental approaches. TSA approaches have proven effective at rapidly screening pseudokinases for ligand binding properties, but it appears difficult to reproduce when the observed change in T$_M$ is low. A previous study analyzing Trb2 in parallel also showed no shift in response to Mg$^{2+}$ or nucleotides, whereas the shift described by Bailey et al. (~2°C) is just above the cutoff for a true binding interaction established previously. Our attempt to reproduce these results with an adapted method showed no change in the T$_M$ of 53Trb22 in response to similar concentrations of EDTA and Mg$^{2+}$, and showed an unexpected decrease in response
when ATP was added. The decreases in response to ATP are small, and it is unlikely to act as a denaturant. Our TSA results therefore agree with Murphy et al.’s finding that Trb2 is insensitive to ATP and Mg\(^{2+}\).\(^{41}\)

It is possible that the discrepancy between our findings and Bailey is attributable to differences in instrumentation. The DSF approach for characterizing pseudokinases described by Murphy et al. (2014) uses a qPCR machine capable of ramping temperature continuously and applied by Bailey et al. (2015), whereas we measured SYPRO fluorescent emission at stepwise 5\(^\circ\)C intervals with extended equilibration times at each step.\(^{40,41}\) This would be less precise than the qPCR approach, as fewer temperatures were measured. However, the largest difference in T\(_M\) observed previously, +6\(^\circ\)C in the presence of EDTA and ATP, should still be resolvable using this method. It is questionable whether or not this shift is actually indicative of ATP binding, as most of the apparent thermostabilization of Trb2 appears dependent on EDTA only. This could suggest that trace metallic cations have a destabilizing effect on Trb2, but this is inconsistent with the fact that Trb2 is unaffected by Mg\(^{2+}\) in the absence of ATP. EDTA is not generally known to directly interact with proteins in solution, although it has been known to introduce error into fluorescent dye-based assays either by stabilizing the native state, or forming complexes with SYPRO at pH’s 9.0 and above.\(^{60,61}\) The “Trb Storage” buffer that was used during binding assays is at pH 9.0, and therefore could have skewed the SYPRO fluorescent emission in the presence of EDTA. This suggests that the established minimum thermal shift for binding confirmation via DSF (\(\geq 3\)^\(\circ\)C) is too low to eliminate false positives.

The circular dichroism melting curve showed much stronger evidence of ATP-binding by Trb2. 53Trb2 secondary structure showed a \(\sim 5\)^\(\circ\)C T\(_M\) increase in the presence of ATP. The large difference in absolute melting temperature measured by CD and DSF is expected, as they
ultimately reflect separate but related unfolding events. DSF measures the exposure of hydrophobic surfaces, which would be driven primarily by the unfolding of tertiary structure elements exposing buried hydrophobic residues. Conversely, temperature-dependent CD measures unfolding of secondary structure elements, which are stabilized primarily by hydrogen bonding interactions. This raises the possibility that ATP is locally stabilizing the secondary structure, but not the global tertiary fold. This could suggest that ATP is weakly interacting with the degenerate nucleotide-binding elements in the N-terminal lobe of the PKD, but the binding is too weak to contribute to the closure of the two lobe domains into the activated ePKD conformation.

MANT-ATP fluorescence showed weak evidence of 53Trb2 ATP binding, producing only a 4% increase in over fluorescence. MANT-ATP fluorescence has been used successfully to demonstrate that HER3 and the JAK2 pseudokinase domain have ATP affinity in the micromolar range.\textsuperscript{62,63} In those cases, energy transfer between MANT and the protein produced 3 – 10 fold increases in fluorescent signal. The relatively small shift in MANT-ATP fluorescence observed could reflect the low ATP affinity of Trb2, which Bailey \textit{et al.} estimate to be in the milimolar range.\textsuperscript{40} However, this would not fully explain the decrease in relative fluorescence in the presence 53Trb2 and MgCl\textsubscript{2}. The differences observed are then likely attributable to experimental noise, and are not reflective of binding between MANT-ATP and 53Trb2.

Overall, no conclusive evidence of ATP binding by Trb2 was found. The approximate Mg\textsuperscript{2+}-independent Kd\textsubscript{[ATP]} reported by Bailey \textit{et al.} of \textasciitilde 0.6 mM approaches the upper limit of detectable nucleotide binding.\textsuperscript{40,41} For comparison, the Kd\textsubscript{[ATP]} of weakly active pseudokinases like JAK2 is on the order 1 – 10 μM. Considering that nearly all intracellular ATP chelates Mg\textsuperscript{2+},
this raises the question that Trb2 and ATP binding can actually occur to any biologically meaningful extent, even if the previously reported interaction was a true positive.

### 4.5 Biological Relevance of Trb2-Nucleotide Interactions

The fact that Trb2 does not appear to bind ATP or catalyze phosphate transfer, with or without Mg\(^{2+}\) is ultimately consistent with what is known about Trb2’s regulatory functions *in vivo*, wherein it has been shown to either coordinate active enzymes non-catalytically or acts as a “decoy” inhibitor for active kinases. If Trb2 inhibits Akt activation as a “decoy kinase” by binding its activation loop in competition with its activators, protein phosphorylation activity *in trans* would be actually be contraindicated to its inhibitory function. Although it is tempting to speculate about autoinhibitory phosphorylation of PEST residues in the NTR, the weak autophosphorylation reported previously (~1% incorporation) is insufficient to explain the global destabilization contributed by the region. Finding catalytic activity where there was previously thought to be none is exciting, as it adds a new dimension to that protein’s signaling functions and opens the possibility of novel drugs targeting the novel catalytic mechanism. Despite the opportunities presented if Trb2 is an active kinase, proceeding under the assumption that it is without clear phenotypic evidence is not a productive route forward.

It is more ambiguous whether or not Trb2 binds ATP, and whether this could serve any regulatory function. It is difficult to distinguish between biologically relevant and vestigial nucleotide binding capacity when characterizing pseudokinases. Pseudokinases are descended either from active kinases that lost activity or pre-catalytic nucleotide-binding proteins. Trb2 features multiple deviations from active kinase sequence affecting nucleotide affinity, implying that the Tribbles homologues became inactive through a stepwise loss of ATP affinity. It then stands to reason that sufficient experimental manipulations could generate some evidence of
vestigial ATP-binding, as the Tribbles homologues only needed need to lose ATP affinity relative to intracellular concentrations of the nucleotide to be rendered inactive in vivo.

**4.6 Conclusions**

Human Trb2 requires both the central PKD and C-terminal C1BD to remain folded and stable above room temperature, and is correspondingly destabilized by the variable NTR region. The 53Trb2 construct forms homodimers and higher-order homomultimers, in contrast to Trb1, its closest homologue. This difference could arise from variations in the intramolecular PKD-C1BD binding surface. Differences in oligomerization could account in part for variations in the signaling potency and effector affinities of the Tribbles homologues.

Despite applying multiple overlapping approaches, previous results of Mg$^{2+}$-independent ATP binding and autophosphorylation by Trb2 could not be observed. Based on our results, it can be concluded that Trb2 is a catalytically dead pseudokinase that does not interact with nucleotides or metallic cofactors at biologically relevant levels. Discrepancies between these findings and the literature illustrate the susceptibility of in vitro pseudokinase characterization to false positives, and that in vivo experiments are necessary as the final arbiters of kinase activity.

**4.7 Future Directions**

**4.7.1 Trb2 Crystal Optimization**

Trb2 remains a promising target for X-ray crystallography. A high-resolution structure would clarify whether the Trb2 nucleotide binding pocket is different enough from Trb1’s to allow for ATP binding. It is not uncommon for large crystals with “good” morphology to have poor diffraction, as is the case with the 53-336 Trb2 crystals. The crystals’ growth speed and reproducibility make them amenable to an expansive optimization strategy, including
microseeding, additive screening, desiccation, side chain modification, and varying growth temperatures. Brute force is also a viable option – it is not uncommon for two crystals in the same drop to diffract differently, so the solution may be just to grow and test enough crystals.

A matrix microseeding (RMMS) approach is also a viable option for Trb2 crystallization, which will be pursued in parallel. In RMMS, crystal seed stocks are applied to divergent crystallization conditions using sparse matrix screens. In traditional microseed experiments, seed stocks are applied to conditions similar to the source condition. This approach with the 53Trb2 spherulites only yielded more spherulites, suggesting that the conditions that favour nucleation are poorly suited to crystal growth. RMMS introduces nucleated protein into broader range growth conditions along with soluble protein, under the assumption that conditions promoting crystal nucleation and growth can be divergent. RMMS has proven successful at producing new hits from hard-to-crystallize proteins, and the ready availability of spherulite seed stocks make it a promising option for Trb2.

4.7.2 Characterization of the Trb2 NTR

Experimental characterization variable Trb2 NTR region is currently limited, due to its destabilizing effect on recombinant Trb2. The PEST sequences are potential targets for inhibitory autophosphorylation, as well as other regulatory post-translational modifications, including proteolysis and ubiquitination. The region could be tested for phosphorylation in trans by treatment with human cell lysate, as well as truncated Trb2. The low concentrations of MBP-fused FL-Trb2 obtainable would also be sufficient to test for PEST dependent proteolysis. The μ and m calpains have been shown to degrade multiple proteins in the endoplasmic reticulum response pathway, in which the Tribbles homologues participate. Calpain digestion of Trb2
would be simple to test for, and identification of the resultant cleavage products would allow for confirmation \textit{in vivo}.

\subsection*{4.7.3 Biophysical Characterization and Crystallization of Trb3}

Human Trb3 remains relatively uncharacterized compared to Trb1 and Trb2. Reports as to the kinase activity of Trb3 are conflicting Bailey \textit{et al.} (2015) present limited evidence of Trb3 autophosphorylation, whereas the only other published \textit{in vitro} data shows total inactivity.\textsuperscript{40,68} This warrants further testing of Trb3’s catalytic activity and nucleotide binding activity. It is also unknown whether or not Trb3 forms a homomultimer like Trb2 or is monodisperse like Trb1. Determination of the Trb3’s oligomerization properties will help elucidate the differences in complex formation between the Tribbles homologues.

Previous attempts to express and purify human Trb3 were marred by poor yield and stability. The construct design and purification strategy used Trb2 should be cross-applicable to obtaining high yields of Trb3 for downstream applications. A Trb3 construct similar to 53Trb2 that omits all but the last 20 residues of the CTR has been designed and cloned into bacterial expression vectors. Following expression and purification, Trb3 will be submitted to the same general characterization and crystallization strategies applied previously to Trb2 for this study. The structure and biophysical properties of the Trb3 Gln84Arg disease variant will also be investigated. This naturally occurring mutant has increased stability and inhibition towards ATF4, linking it to cardiovascular disease and insulin resistance.\textsuperscript{69} Understanding the structural differences introduced by the Gln84Arg polymorphism could reveal novel drug targets for the treatment of related disease states.
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## Appendix A

### Trb2 Expression Optimization

Table A Conditions screened for Trb2 Expression and Solubility

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Legend

RI – Roche protease inhibitor FT – Freeze-thaw NL – Natural lysis (lysozyme) TX – Triton-X 100
Appendix B

Trb2 Crystallization Screens

The following commercially available crystallization condition screens were dispensed to 96-well SDVD plates and used to test for the crystallization of Trb2 as described in Materials and Methods.

Table B 96-Well SDVD Conditions Screened for Trb2 Crystallization

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