2009

ELUCIDATION OF THE MOLECULAR MECHANISMS OF ACTION OF PINI

Melanie L. Bailey

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(Spine Title: Elucidation of the Molecular Mechanisms of Action of Pin1)

(Thesis Format: Integrated Article)

by

Melanie L. Bailey

Graduate Program in Biochemistry

A thesis submitted in partial fulfillment for
The degree of Doctor of Philosophy

School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, ON Canada

Abstract

The enzyme Pin1 is a peptidyl-prolyl isomerase (PPIase) that structurally consists of an N-terminal WW interaction domain and a C-terminal catalytic or PPIase domain. The understanding of how these two domains work together and perform the many functions discovered in cells is incomplete. Therefore, we hypothesize that Pin1 acts as an extra regulatory step in signalling pathways by first binding to targets with its WW domain and then catalyzing the isomerization of those same proteins using its PPIase domain.

To gain insights into how Pin1 performs its molecular function, we mutated specific residues of Pin1 and examined both their ability to support viability in yeast and isomerase activity. We determined that in the phospho-specific binding loop of the PPIase domain K63 was the most important basic residue for Pin1 function and activity while only one contact from R68 or R69 was needed. Furthermore, by mutating the Pin1 catalytic residue, C113, to both D and S, we showed that a negative charge at this position is more important than a nucleophile. Finally, extensive mutagenesis of two conserved active site histidines, H59 and H157, determined that these two residues are not as important as C113 for Pin1 function or activity. Instead, protein stability experiments suggested a structural role for both histidines. Collectively, these results led us to propose a new non-covalent mechanism for Pin1 catalysis.

We also examined the binding of Pin1 mutants to full-length targets and surprisingly found that a binding-deficient mutation in the PPIase domain, R68/69A, had a lower affinity for most Pin1 targets. We also discovered two classes of Pin1 binding proteins: Class I proteins bind both the WW and PPIase domains of Pin1 while Class II

proteins mainly bind the WW. We proposed, based upon structures of the WW and PPIase domains bound to peptides, that the differences between Class I and Class II binding may relate to the presence of a proline at the +1 position. These results have provided novel insights into the binding of the two domains of Pin1 to full-length targets. Taken together, these studies have expanded the knowledge of the molecular mechanisms of Pin1 action in cells.

Keywords: Pin1, peptidyl-prolyl isomerase, isomerization, non-covalent catalysis, Cdc25C, NonO, MPM-2 antibody, bond distortion, protein stability, phosphorylated serine/threonine-proline motif.

Coauthorship

Chapter 2:

The mutants Y23F, K63A, R68A, and R69A were generated by Norclone (London, Ontario, Canada). Single-site mutants R69W and H59Q were obtained during unigenic evolution performed by Colleen Behrsin. The Y204 constructs for Y23A, R68/69A and C113S were generated by Kathy Bateman.

The original unigenic evolution study, of which Figure 2.2 is a summary, was performed by Colleen Behrsin.

The *in vitro* isomerase assays in Figure 2.4 and 2.5 were performed by Dr. Brian Shilton (Shilton Laboratory, University of Western Ontario)

Chapter 4:

NonO and SFPQ were identified after trypsin digest and MALDI-TOF analysis. Trypsin digest was performed by Victoria Clarke and Kristina Jurcic (Functional Proteomics Facility, University of Western Ontario). Kristina Jurcic also performed MALDI-TOF for the data used for Supplementary Figure 4.1.

Acknowledgements

I would first like to thank my supervisor, Dr. David Litchfield, for all of his help and support while I was working in his laboratory. I was always grateful to have a supervisor as excited by my project as I was. I would also like to thank my committee Dr. Christopher Brandl and Dr. Brian Shilton for all of their comments and suggestions over the years. Throughout my time in the Litchfield Lab, I have learned much from each and every one of its members including Dr. Mary Olsten, Rich Derksen, Ashley French, Dr. David Canton, Dr. James Duncan, Nicole St.-Denis, Erin Parker, Dr. Kelly Duncan, Dr. Laszlo Gyenis, Jake Turowec, Kathryn Garside and Dr. Greg Vilk. You can never be truly lonely on the Pin1 project. I would especially like to thank Mary, Ashley and Nicole for good talks in the office, scientific or otherwise. And I am especially grateful to Erin for coming and working on Pin1.

I am also grateful to members outside of the Litchfield Lab who were there to help me with my experiments including members of the Brandl and Shilton laboratories for help with methods and equipment usage. Lee-Ann Briere in the Biomolecular Interactions and Conformations Facility and Victoria Clarke and Kristina Jurcic in the Functional Proteomics Facility all provided help for aspects of this thesis.

Finally, I am grateful to my family for always being there in the past five years. Specifically, I would like to thank my parents for listening even when they did not understand the details and especially I would like to thank my sister, Colleen, a fellow explorer in the frontiers of science.

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List of Abbreviations, Symbols and Nomenclature

AD	Alzheimer's Disease
APP	Amyloid precursor protein
CD	Circular dichroism
Cdc25C	Cell division cycle 25, isoform C
CDK	Cyclin-dependent kinase
CyP	Cyclophilin
Ess1	Essential-1
FKBP	FK506-binding protein
5-FOA	5-fluoroorotic acid
GSK	Glycogen synthase kinase
GST	Glutathione-S-transferase
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MAPK	Mitogen-activated protein kinase
MPM-2	Mitotic protein monoclonal #2
NonO	Non-POU domain-containing octamer binding protein
NP-40	Nonidet P-40
OD	Optical density
Par14	Parvulin, 14kDa
PBS	phosphate buffered saline
Pin1	Protein interactor of NIMA
Plk1	Polo-like kinase-1
Pol II CTD	RNA polymerase II C-terminal domain

PP2A	Protein phosphatase 2A
PPIase	Peptidyl-prolyl isomerase
pS/T-P	phosphorylated serine or threonine residue followed by a proline
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
TEV	Tobacco etch virus
μg/μL, mg/mL	microgram/microlitre, milligram/millilitre

Chapter 1: Introduction

1.1 General Introduction

Regulation of important processes in the cell is controlled by post-translational covalent modifications like phosphorylation. As it is a reversible process, the phosphorylation profile of the proteome changes dynamically depending on the state of the cell with an estimated 30% of proteins being phosphorylated at any one time (*1*). The drastic changes seen in global phosphorylation in many diseases like cancer emphasize how control of this modification is essential for normal cell function. For this reason, the study of proteins that bind to and/or regulate phosphorylation is of major interest. The set of enzymes that directly modify and control phosphorylation, protein kinases and protein phosphatases, are well-known and have been extensively investigated because disruption of their balance changes the phosphorylation status of the cell and can lead to diseases like cancer (*2, 3*). More recently, however, the enzyme Pin1 has been proposed as another layer of regulation for phosphorylation as it has been shown to specifically bind and isomerize phosphorylated sequences (*4-7*). The number of targets of Pin1 is still growing (*8, 9*) and many show important and diverse cellular function suggesting that Pin1 is critical in the various phosphorylation-regulated pathways in the cell.

1.2. General classification of Pin1 in the PPIase family

Due to their partial double bond character, the peptide bonds that connect individual amino acids in proteins have the potential to exist in two conformations, *cis* and *trans* (Figure 1.1A) *(10)*. Although almost all peptide bonds are found in the *trans* conformations in proteins, those that precede a proline are unique and may exist in the *cis*

conformation up to 30% in peptides and in 10% of protein structures (*11, 12*). The uncatalysed chemical interconversion between *cis* and *trans* is a relatively slow process and must overcome a high activation energy barrier of around 16-22kcal/mol (*13*). The rate of rotation depends on such factors as structural constraints in the peptide or protein and the identity of the amino acid preceding the proline. The addition of a phosphate to a threonine peptide, for example, decreases the rate of isomerization more than seven times (*14*). As the rate of isomerization is so intrinsically slow, a class of enzymes termed the peptidyl-prolyl isomerases (or PPIases) has evolved to help speed up this reaction to a cellular time scale (*10*).

Three main groups of PPIases have been discovered to date: the Cyclophilins (CyPs), FK506-binding proteins (FKBPs) and the parvulins (Figure 1.1). The CyPs and FKBPs were discovered earlier than the parvulins because of their ability to bind immunosuppressive drugs (eg. Cyclosporin A and rapamycin). Both groups were determined to perform the same isomerization reaction even though they shared no similarity in their sequence (*15-17*). Later structural analysis determined that they also did not share the same structural fold (*18, 19*). CyP PPIase domains were found to be folded into an 8-stranded β-barrel structure and FKBPs formed their own distinctive α/β fold. More than 16 homologues have been discovered for both the CyPs and FKBPs in humans, most of which have been shown to have protein folding and chaperone functions (*20*).

Upon its discovery in yeast in 1989, Pin1 was immediately placed in its own distinct group of PPIases as it was found to be unresponsive to the immunosuppressive drugs that target the CyPs and FKBPs (*21-23*). As orthologues in other organisms were

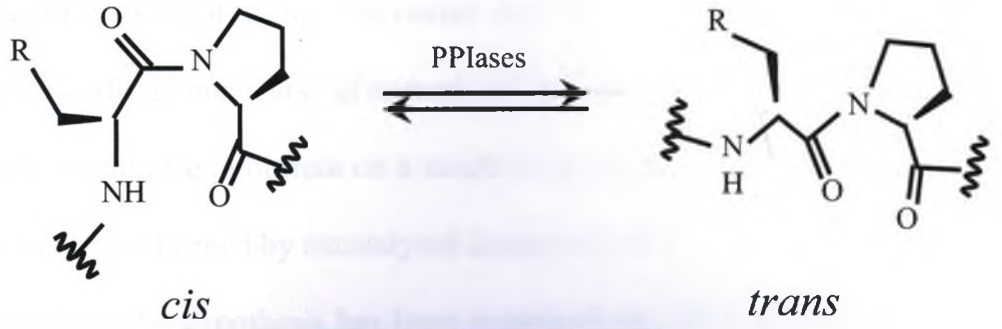
found, it became apparent that this new family of PPIases had both unique sequence and structural characteristics (*23*). Given the name the parvulin family (meaning very small) (*23*), this group of enzymes is generally divided into two groups based on their recognition sequence preference: 1) the non-Pin1 parvulins (eg. Par14) which can isomerize the peptide bond between an unphosphorylated amino acid and a proline; and 2) the Pin1-like parvulins which specifically recognize motifs with a phosphorylated serine or threonine residue followed by a proline (pS/T-P). It should be noted that while the non-Pin1 parvulins can be found in all complexities of organism, Pin1-like parvulins have only been discovered in eukaryotes including yeast, *Drosophila*, plants and mammals (*21, 24-26*). Sequence identity between human Pin1 and the Pin1 homologue, Ess1 in *S. cerevisiae* is 45% while identity between human Pin1 and Par14 (the only other parvulin discovered in humans) is 34% (*21, 27*). A sequence alignment of parvulins from different organism can be found in Chapter 3. Similarly, only Pin1-like parvulins are functionally conserved, able to compensate for the loss of the Pin1 homologue, Ess1, in yeast (*21, 25, 28*).

1.3 Pin1 functions in cells: a possible therapeutic target

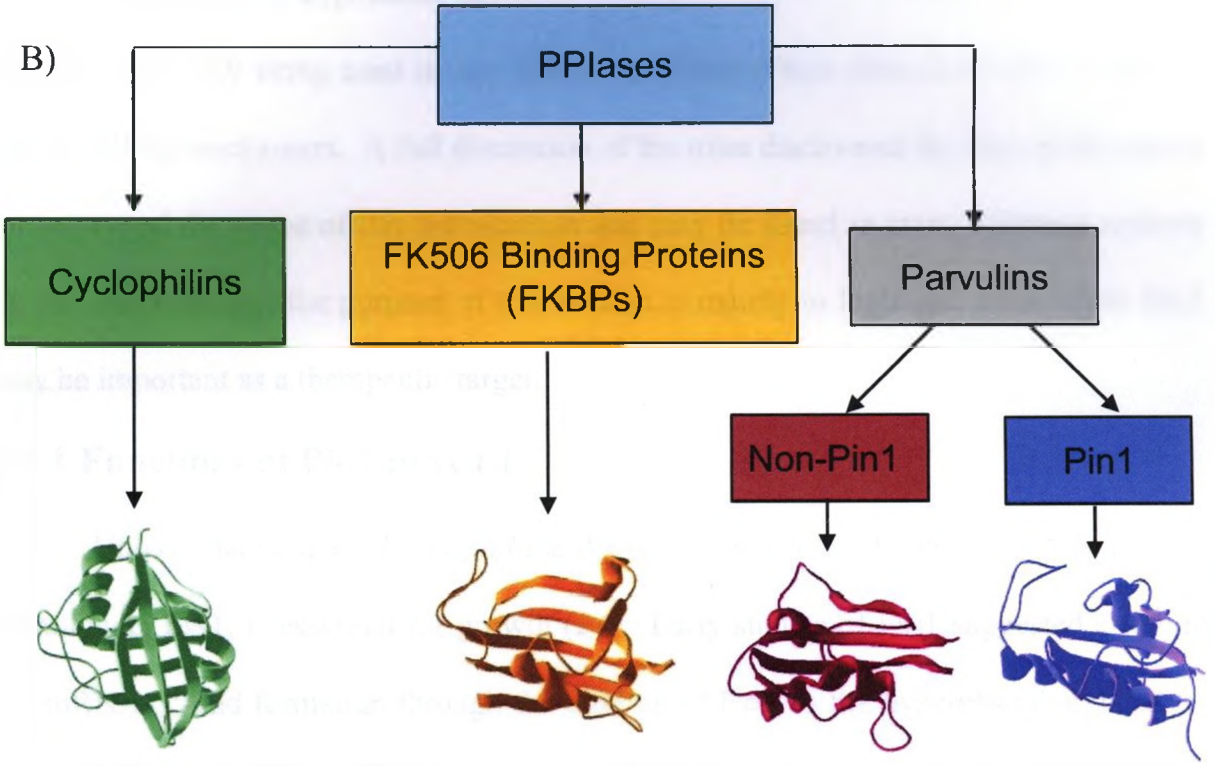
As an isomerase, the main role of Pin1 is in maintaining equilibrium between the *cis* and *trans* isomers of the protein; it does not push the reaction in one direction exclusively. As such, one might not expect the protein to contribute significantly to cellular signalling networks. This expectation is unfounded, however, as Pin1 has been shown to affect many cellular proteins of diverse functions (*8, 9*). Moreover, Pin1 itself has been shown to be necessary for numerous cellular processes and changes in its expression are involved in disease. Generally, this dichotomy is reconciled by the idea

Figure 1.1- Pin1 is a member of the peptidyl-prolyl isomerase (PPIase) class of enzymes. A) The isomerization reaction catalyzed by all PPIases. PPIases differ in the R group they recognize on the amino acid prior to the proline. Pin1 recognizes a pS or pT in this position. B) Breakdown of the PPIases into three separate groups based on their sequence similarity. Pin1 is a member of the parvulin group of PPIases and shares a similar structural fold with the FK506 binding proteins (FKBPs) but not the cyclophilins (CyPs). Structural representations of each type of domain are shown: CyP18 (PDB: 2CPL), FKBP12 (PDB: 1FKB), hPar14 (PDB: 1EQ3), Pin1 (PDB: 1PIN). C) A comparison of various traits amongst different PPIases.

A)



B)



C)

Feature	CyP 18	FKBP12	hPar14	Pin1
PPIase domain size	~18kDa	~12kDa	~10kDa	~10kDa
PPIase domain fold	8-strand β-barrel	α/β FKBP superfold	α/β FKBP superfold	α/β FKBP superfold
# homologues in humans	>16	>16	1	1
Specificity for R group	Small hydrophobic	Large hydrophobic	Basic residues	pS or pT
Function	Protein folding	Protein folding	Unknown	Signalling
Physiological inhibitors	Cyclosporin A	FK506, rapamycin	PiB	Juglone, PiB

that although Pin1 cannot change the isomer equilibrium, the pools of these two isomers may change rapidly in the course of normal cell signalling. Therefore, Pin1 is needed for the reestablishment of equilibrium on a much faster, more physiologically relevant, time scale than can be performed by uncatalyzed isomerization.

Therefore, the hypothesis has been presented that Pin1 may act as a "molecular timer", potentially being used in any cellular pathway where phosphorylation is part of the signalling mechanism. A full discussion of the roles discovered for Pin1 in the cell so far is beyond the scope of this introduction and may be found in many literature reviews (*8, 9*). Accordingly, the purpose of this section is mainly to highlight areas where Pin1 may be important as a therapeutic target.

1.3.1 Functions of Pin1 in yeast

Unlike mammalian, *Drosophila* and even *S. pombe* Pin1, the *S. cerevisiae* Pin1 homologue, Ess1, is essential for growth (*24*). Early studies of Ess1 suggested a role in pre-mRNA 3'-end formation through the binding of Ess1 to the hyperphosphorylated C-terminal domain (CTD) of RNA polymerase II, the main transcirber of mRNA (*29-31*). Additionally, multicopy suppressors that rescue the *ess1* lethal phenotype included genes important for Pol II transcription and regulation (*32*). Later, studies confirmed that Ess1 could enhance the dephosphorylation of serine 5 in the CTD by CTD phosphatases in a manner that may play a role in transcription initiation (*33, 34*).

The essential role of Ess1 in yeast is complicated by more recent results by Gemmil *et. al* (2005) which showed that yeast need only a small amount of Ess1 (less than 400 molecules) to maintain growth under optimal conditions (*35*). As they found that yeast did require more Ess1 under conditions of stress, Gemmil *et. al* suggested that

in yeast, the essential function of Ess1 may be to facilitate transcription of a small number of Pol II-dependent genes- likely including those that are induced by Pol II in response to stress (*35*). Overall, these results suggest an essential transcriptional role for Ess1 in yeast viability which is not seen for the non-essential human Pin1, making Ess1 a possible anti-fungal target.

1.3.2 The role of Pin1 in mammalian cell growth

Due to its ability to bind motifs phosphorylated by Pro-directed kinases, enzymes that are often involved in the regulation of cell growth and proliferation (eg. CDKs, MAPKs), Pin1 was initially characterized as a mitotic regulator (*21*). Although many other functions for Pin1 have since been shown, there still remains a robust role for Pin1 in the regulation of cell growth.

Firstly, Pin1 is involved in a number of essential cell cycle processes. For example, one recent study showed that Pin1 can regulate centrosome duplication in the cell in S phase, although the mechanism behind this observation is still unknown (*36*). Furthermore, Pin1 also localizes to chromatin in G2/M cells and it can induce chromatin condensation *in vitro* through interaction with Topoisomerase IIα (*37*). Pin1 also helps coordinate the S and M phases of the cell cycle through stabilization of early mitotic inhibitor-1 (Emi1) (*38*) and has been shown to enhance growth signalling pathways often through the stabilization of transcription factors such as c-Jun and β-catenin (*39, 40*).

In addition to the evidence above, other studies have shown that the misregulation of Pin1 can lead to cell cycle perturbations at important checkpoints. Originally, Pin1 overexpression in HeLa cells was shown to lead to an arrest at the G2/M checkpoint while depletion of Pin1 resulted in cells arrested in M phase (*21*). Later work in

mammalian cells has shown that Pin1 depletion can lead to a decrease in proliferation and even cell death (*41*). As well, Pin1 knockout mouse embryonic fibroblasts display defects in the G0-G1 transition (*42*) and Pin1 knockout primordial germ cells had a longer cell cycle due to a prolonged G1-S transition (*43*). Knockout mice appear to develop normally, but have been found to have a number of proliferative defects including retinal and testicular atrophy and lack of breast epithelial expansion during pregnancy (*44*). Although there are some discrepancies, these studies all point to a role for Pin1 in cell growth due to its involvement in key cell cycle processes and transitions.

1.3.3 Abnormal regulation of Pin1 in pathogenesis

Given its many roles in the cell, especially its role in growth and proliferation, it is not surprising that aberrant Pin1 function in cells has been found in human disease. Both increased and decreased Pin1 activity can lead to pathogenesis emphasizing its potential as a therapeutic target.

a) Overexpression of Pin1 in cancer

There have been a number of studies that have shown a role for Pin1 in oncogenesis. First, Pin1 has been shown to be overexpressed at both the mRNA and protein level in many, although not all, tumour types including breast, prostate and colorectal cancer (*40, 45-47*). In a study by Bao *et. al* of 2041 tumour samples representing 60 tumour types, 38 showed overexpression of Pin1 protein in more than 10% of cases as compared to normal controls (*45*). Levels of Pin1 correlate well with disease progression in prostate, colorectal and lung cancers (*46-48*) and prostate cancer patients with higher levels of Pin1 have higher levels of recurrence (*46*). In mice, transgenic overexpression of Pin1 in the mammary glands causes mammary hyperplasia

and eventually tumourigenesis (*36*). By comparison, depletion of Pin1 suppresses tranformation induced by oncogenic Neu or Ras (*49*).

As Pin1 can interact with multiple cell cycle regulators and oncogenes (see Section 1.5.1), it is likely that there is more than one mechanism through which Pin1 overexpression can increase oncogenesis. One mechanism that has been studied extensively, however, is the Cyclin D1/Pin1 positive feedback loop (Figure 1.2A). Through interaction with the pT286-P motif on Cyclin D1, Pin1 can increase the stability of Cyclin D1 and enhance its accumulation in the nucleus (*44*). Nuclear Cyclin D1 then facilitates E2F transcription through inhibition of the Rb protein which allows progression through the G1-S checkpoint of the cell cycle. As an E2F target gene, Pin1 expression is then increased enabling further stabilization and nuclear accumulation of Cyclin D1 (*49*). Pin1 can also affect Cyclin D1 levels indirectly through the stabilization of the transcription factors c-Jun, β-catenin and NFκB (*39, 40, 50*). There is evidence that this positive feedback loop may indeed be deregulated in cancer, as clinical studies have shown that Pin1 expression in certain tumours positively correlates with Cyclin D1 expression (*40, 51, 52*). Other Pin1 interactions which may enhance its role in oncogenesis include those with Focal adhesion kinase (FAK), Akt/PKB and Wee1 (*53-55*). Studies have also suggested that Pin1 suppression may be a way of targeting this molecular pathway which is often uncontrolled in human cancer (*56, 57*).

b) Loss of Pin1 in neurodegeneration

The involvement of Pin1 loss in neurodegeneration, and more specifically its contribution to Alzheimer's disease (AD), appears to be almost a direct contrast to its involvement in cancer. Much evidence has been presented for a role for Pin1 in AD as it

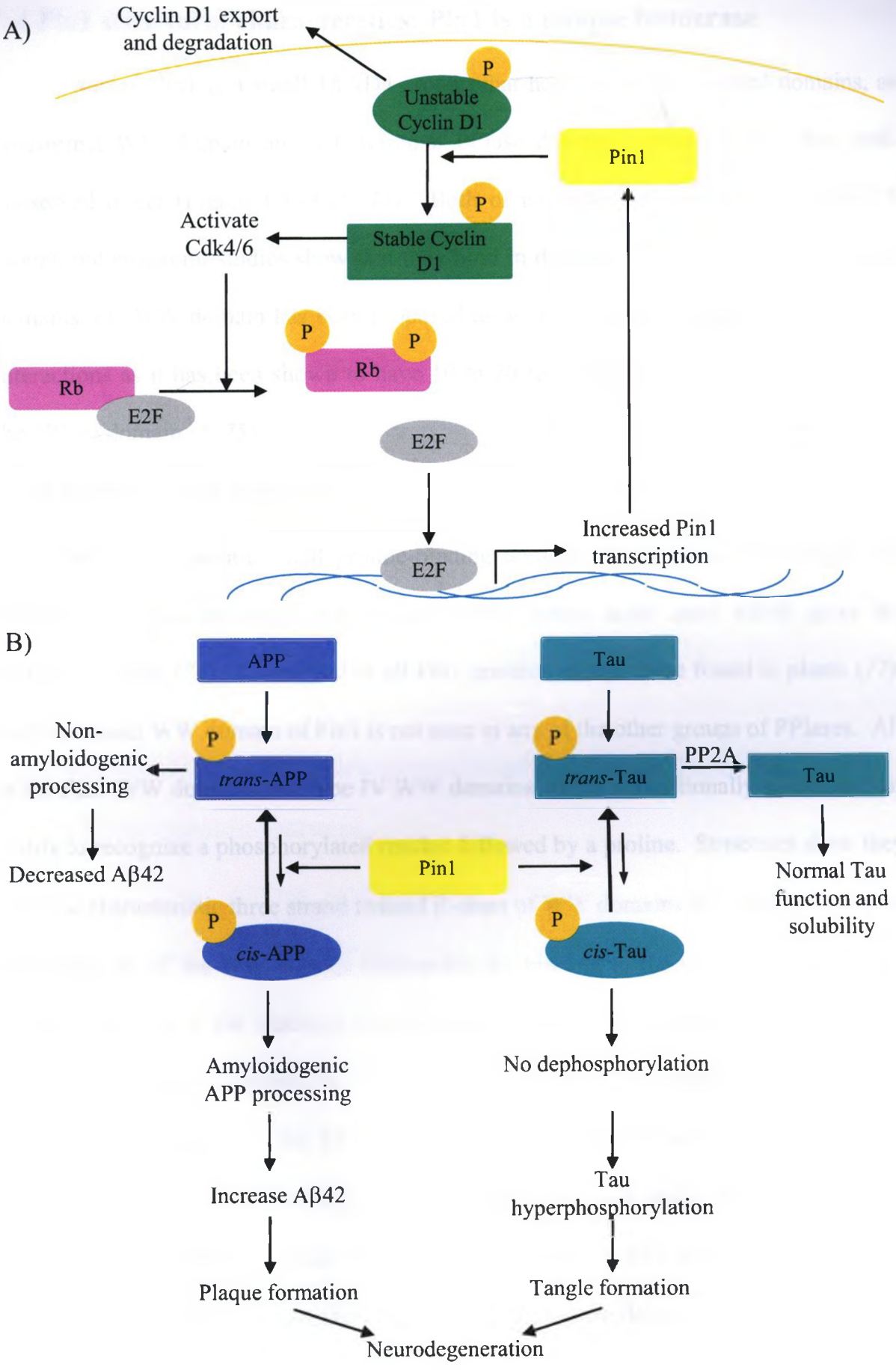
has been found to contribute to both of the two main pathologies of AD: neurofibrillary tangles composed of hyperphosphorylated Tau and senile plaques which contain insoluble Aβ peptides (Figure 1.2B). Initially, some of the most convincing evidence of a link between Pin1 and AD was demonstrated in Pin1 knockout mice. These mice were found to develop age-dependent motor defects and behavioural deficits accompanied by hyperphosphorylated Tau, Tau filament formation and neuronal degeneration (*58*). Later, Pin1 knockout mice were also shown to have age-dependent accumulation of insoluble Aβ peptides in the brain (*59*). In normal human brains, Pin1 expression has been found to inversely correlate with predicted neuronal vulnerability to degeneration (*58*). In AD, Pin1 expression and localization shows variability. For example in different studies, it has been found to be either less soluble in AD brains (*60*), colocalized with phosphorylated Tau granules in AD brains (*61*) or expressed at lower levels in the degenerated areas of AD brains (*58*). In all of these cases, however, Pin1 expression or localization was found to differ from normal brains, suggesting that Pin1 may be important for protection against neurodegeneration.

Evidence for the involvement of Pin1 in AD neuropathology has been consistent on a molecular level, as Pin1 has been found to interact with both Tau and amyloid precursor protein (or APP- the precursor for Aβ peptides) (Figure 1.2B). Pin1 interacts with Tau phosphorylated on T231 and likely on T212 (*60, 62*). Interaction of Pin1 with pT231 leads to its dephosphorylation by the *trans*-specific phosphatase PP2A and allows Tau to bind to microtubules and perform its normal function (*60, 63*). Alternatively, if Pin1 is not allowed to perform its function, Tau would not be dephosphorylated leading to its characteristic accumulation in fibrillary tangles (*8*). In the case of APP, the *cis* and

trans isoforms of the protein have been proposed to lead to different types of APP processing with the *cis* form undergoing amyloidogenic processing and increasing the insoluble Aβ peptides seen in plaques in AD (*59, 64*). Interaction of the pT668-P site of APP with Pin1 has been suggested to lead to non-amyloidogenic processing and decreasing Aβ peptide production (*8, 59*). That Pin1 directly interacts with two of the pathogenic proteins involved in AD may explain why Pin1 is needed to maintain normal neuron function.

Collectively, these studies show that Pin1 has important roles in both yeast and mammalian cells and that studies that target Pin1 at the molecular level may help in determining its therapeutic potential in diseases such as cancer and AD. Consequently, several Pin1-based inhibitors have been developed, but are limited by their non-specificity or cell impermeability. The drug juglone, for example was originally discovered to inactivate parvulin-like PPIases through interaction with the catalytic cysteine (*65*). Juglone has since been shown, however, to inactivate other cysteine-rich proteins in the cell (*66, 67*). Several other studies have taken structure-based approaches in their design of Pin1 inhibitors (*68-72*). Unfortunately, these inhibitors contain a phosphate group to enhance their affinity which then makes these molecules less membrane permeable. Encouragingly, Pin1 RNAi has shown that the downregulation of Pin1 blocks cancer cell growth and proliferation (*56*) and the development of more specific Pin1 inhibitors may be aided by a better molecular understanding of the regions of Pin1 needed for function.

Figure 1.2- The involvement of Pin1 in pathogenesis. A) A positive feedback loop involving Cyclin D1 in cancer. Pin1 acts on pT286 of Cyclin D1 causing its nuclear accumulation and stabilization. Cyclin D1 then partners with its Cdk and phosphorylates targets such as Rb. This results in the release of E2Fs allowing transcription of E2F genes of which Pin1 is one. Higher Pin1 expression can the stabilize more Cyclin D1 creating a positive feedback loop. B) Interaction with Tau and APP in neurodegeneration. Pin1 can bind to both the pT668 site on APP and the pT212/231 sites on Tau, rapidly establishing equilibrium between the *cis* and *trans* isomers. In both cases, the *trans* form of the substrate is necessary for normal signalling and healthy neuron function. The *cis* forms of these substrates cause the accumulation of plaques, in the case of APP, or tangles, in the case of Tau- both of which are pathological hallmarks of neurodegeneration. Figure adapted from Lu & Zhou (2007).


A)
Cyclin D1 export and degradation
P
Unstable Cyclin D1
Pin1
P
Activate Cdk4/6
Stable Cyclin D1
P
P
Rb
Rb
E2F
E2F
E2F
Increased Pin1 transcription
B)
APP
Tau
Non-amyloidogenic processing
P
trans-APP
P
trans-Tau
PP2A
Tau
Decreased Aβ42
Pin1
Normal Tau function and solubility
P
cis-APP
P
cis-Tau
Amyloidogenic APP processing
No dephosphorylation
Increase Aβ42
Tau hyperphosphorylation
Plaque formation
Tangle formation
Neurodegeneration

1.4 Pin1 structural characterstics: Pin1 is a unique isomerase

Human Pin1 is a small 18 kDa protein that has two well-conserved domains, an N-terminal WW domain and a C-terminal PPIase domain, connected by a less well-conserved linker (Figure 1.3A) (*5, 73*). Both of these domains can recognize pS/T-P motifs, but structural studies show that they bind in different ways (*69, 74*). Of the two domains, the WW domain has been proposed to be predominantly responsible for target interactions as it has been shown to have 10 to 20 fold higher affinity for peptides than the PPIase domain (*6, 75*).

1.4.1 WW domain features.

WW domains are small proline-binding domains 35-40 residues in length and contain two invariant tryptophan residues 20-22 amino acids apart which gives the domain its name (*76*). Conserved in all Pin1 proteins except those found in plants (*77*), the N-terminal WW domain of Pin1 is not seen in any of the other groups of PPIases. All of the Pin1 WW domains are Type IV WW domains which is functionally based on their ability to recognize a phosphorylated residue followed by a proline. Structures show they have the characteristic three strand twisted β-sheet of WW domains and that there are two major regions of the WW domain responsible for binding to the pS/T-P motifs (Figure 1.3B). The first is the stacking interactions between Y23 and W34 of Pin1 and the proline of the binding partner (*74, 78*). The second source of binding on the WW domain is the loop between β1 and β2, also called the "p patch", which co-ordinates the phosphate moiety. Structures suggest that the most important single interaction of the p patch is the electrostatic contact between the side chain of R17 and the oxygen of the phosphate (*74*), but NMR data shows that this loop is quite flexibile and may undergo a

conformational change upon binding of ligand (*79, 80*). This loop is longer by one residue in Pin1 than in other groups of WW domains and a shorter loop cannot bind pS/T-P motifs (*81*) suggesting that the conformation of this loop is also important in accommodating the larger phosphate group.

Structures of peptides bound to the WW domain reveal that the bound peptides themselves are in an extended conformation and the N- and C-termini are always in the same orientation (*74*). With regards to the conformation of the proline of the bound molecule, data using peptides derived from Cdc25C, Tau and the RNA polymerase II C-terminal domain (Pol II CTD) suggest that the WW binds motifs in the *trans* conformation (*74, 78*); however, recent NMR work with a small peptide derived from APP shows it can bind in the *cis* conformation (*59*) even though previous structural modelling suggested this would be inhibited by steric constraints (*74*). This conflict has yet to be resolved and an extensive understanding of how the WW domain alone can bind to proteins would help in determining binding mechanisms for full-length Pin1.

1.4.2 PPIase domain features

Pin1 PPIase domains exhibit sequence homology to other parvulin PPIase domains and share their overall structural fold with both parvulins and FKBPs (*5, 23, 82*). Various structural studies have localized the active site of Pin1 in the concave side of the middle of the central β-sheet, slightly enclosed by helix α4. Individual residues involved in PPIase domain binding and catalysis have been further refined by mutational studies with the most important residues being highlighted here (Figure 1.3B):

First and most well-conserved is the proline binding pocket. Consisting of at least three hydrophobic residues (L122, M130 and F134 in human Pin1), this pocket is

believed to be responsible for clamping onto the proline in the substrate and likely holding it in place during catalysis (*5, 69, 83*). Similar hydrophobic pockets have been found in all parvulin structures so far (*5, 26, 84-89*). As well, it has been suggested that both CyPs and FKBPs possess a similar hydrophobic pocket and that this may be the one common structural feature shared by all PPIases (*82*).

On the opposite side of the active site lies the phosphate-specific binding loop. This loop between the β4 and α1 of the PPIase domain is conserved in only the Pin1-like parvulins (*21, 24, 25, 77*) and contains three basic residues, K63, R68 and R69 in human Pin1, which likely coordinate the acidic phosphate group of the substrate. Various studies have suggested that this loop has a certain amount of flexibility which may allow for accommodation of rotation around the peptide bond during catalysis (*74, 83, 90*).

The region between the proline binding pocket and phosphate-specific binding loop accommodates the peptide bond of the substrate that needs to be rotated. Residues in this middle part of the active site include H59 and H157 which flank the peptide bond from the bottom of the active site as well as C113 whose sulphydryl group lies above the carbonyl oxygen of the substrate bond in the original crystal structure of Pin1 (*5*). Both H59 and H157 are well-conserved in all parvulins (both Pin1 and non-Pin1), but their exact role in Pin1 function and catalysis has yet to be determined. C113 is conserved as either a C or a D in parvulins and reasons for why such drastically different residues are tolerated at this position are examined further in Chapter 2. Given the position of C113 above the substrate carbonyl, it has been postulated to be an important catalytic residue. This appears to be true as mutation of C113 to alanine or serine has been shown to decrease Pin1 PPIase activity to 6 or 4% of wild-type PPIase activity *in vitro (63)*.

Structures of Pin1 with substrates in its PPIase domain have been difficult to obtain, perhaps because of the high catalytic efficiency of Pin1 or because of the relatively low binding affinity of the PPIase domain compared with the WW domain. The original crystal structure of Pin1 had an A-P dipeptide found in the *cis* conformation in the active site (*5*). It has subsequently been demonstrated, however, that there must be three peptide bonds for a substrate to be isomerized by Pin1 and that peptides with fewer bonds act as low affinity inhibitors of the PPIase domain (*68*). A more recent structural study has examined crystals of two related pT-P-containing peptide inhibitors in the active site of Pin1: one inhibitor with the pT in the D conformation was found in the active site in *trans* and the peptide with pT in the L conformation was found in *cis (69)*. These structures show that the Pin1 active site can accommodate peptides in either the *cis* or *trans* conformation with relatively little structural rearrangement in its active site; however, the structure of the transition state of substrates has yet to be determined.

1.4.3 Linker function and domain interactions

The linker connecting the WW and PPIase domains is the least well-conserved part of Pin1. It varies in length, sequence and structure. In human Pin1, the linker is short and unstructured (*5, 73*), but structures of Pin1 homologues in *Candida albicans* and *Aspergillus nidulans* have shown longer linkers which contain an α-helix (*79, 85*). It has been proposed that these structured linkers may be fungal-specific, rigidly orienting the WW and PPIase domains to bind important fungal targets such as the RNA polymerase II C-terminal domain (Pol II CTD) (*85*). In human Pin1, however, the WW and PPIase domains interact only weakly in solution (*91*) suggesting that the more flexible linker of Pin1 may allow for broader substrate specificity (*85, 91*).

Overall, structural studies have determined how each of the two domains of Pin1 bind short phosphorylated peptides. But it is still unknown how Pin1 binds full-length proteins and how the two domains of Pin1 interact to perform their function *in vivo*.

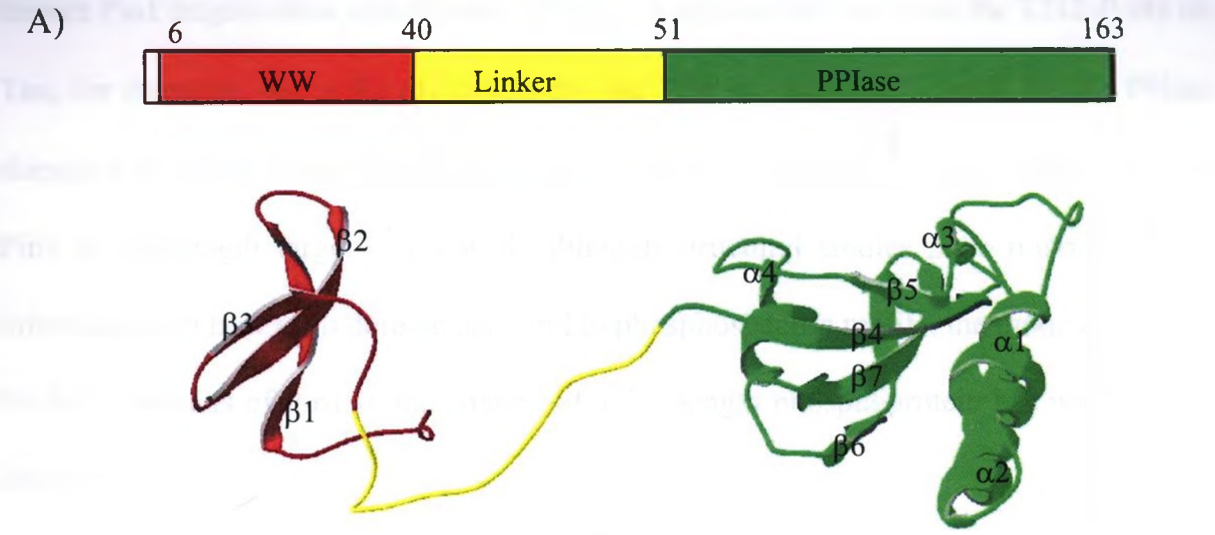
1.5 Molecular mechanisms of Pin1 action: the binding vs. Catalysis debate

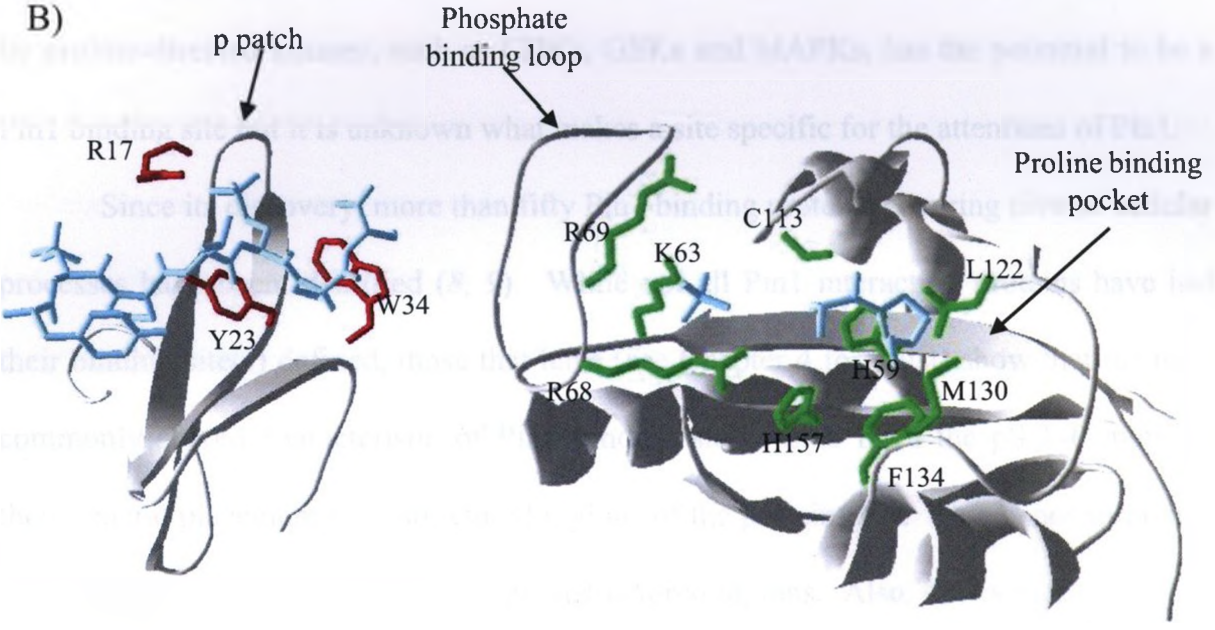
Unlike phosphorylation and other covalent modifications, isomerization is especially hard to detect *in vivo* because the atom content of the substrate protein remains unchanged. To this end, although PPIases can perform catalysis on peptide substrates *in vitro*, strong evidence that their isomerase activity is needed for their function *in vivo* has yet to be presented and still remains an unresolved issue even for well-studied PPIases like Pin1 (*92*). A complete understanding of how Pin1 binding and activity is involved in its many cellular functions is needed if we are to fully understand its role as a regulator of phosphorylation-dependent processes.

1.5.1 Pin1 as a phosphospecific interacting protein

The ability of Pin1 to interact with phosphoproteins, mainly through its WW domain, has been well-documented (*4, 6-9*). That Pin1 can immunoprecipitate interactors in a phospho-dependent manner and colocalize with structures, such as those containing phosphorylated Tau (*61*), lends support to its role as a phosphospecific binding protein *in vivo*. Early studies determined that optimal binding for Pin1 was to peptides that had large hydrophobic and aromatic residues surrounding the critical pS/T-P motif (*4*). Lu and colleagues determined that Pin1 binding to an analogue of the optimal Pintide peptide (WFYpSPFLE) showed two dissociation constants (K_ds) of 1.2μM and 11μM for the WW and PPIase domains, respectively (*6*). However, studies using peptides derived from

Figure 1.3- Structural features of Pin1. A) Pin1 is composed of a WW and PPIase domain separated by a flexible linker. PDB: 1NMV. B) Important residues in the WW (left) and PPIase (right) domains. Backbones of each domain are shown in grey ribbons while selected residues for the WW domain are displayed in red and for the PPIase domain in green. Molecules bound in the crystal structures of the two domains are in blue. The WW domain was crystallized with a peptide based on the RNA polymerase II C-terminal domain (PDB: 1F8A). The crystal structure of the PPIase domain contains both an A-P dipeptide and a sulphate ion in its active site (PDB: 1PIN).


A)
6
40
51
163
WW
Linker
PPIase
β2
β3
β1
α4
α3
β5
β4
α1
β7
β6
α2


B)
p patch
Phosphate
binding loop
R17
W34
Y23
R69
K63
C113
Proline binding
pocket
L122
H59
R68
M130
H157
F134

known Pin1 targets show much lower affinity. A peptide derived from the T212-P site on Tau, for example, had a K_d of 160μM for the WW domain and 3.3mM for the PPIase domain (*75*) and it is not known how these studies with peptides relate to the binding by Pin1 to full-length targets. As well, although structural studies have provided much information on how each domain can bind to phosphospecific motifs, the mode of binding for both domains of Pin1 in the context of a full-length phosphoprotein has not yet been determined.

a) Proline-directed phosphorylation and Pin1 binding

Phosphorylation of a serine or threonine residue followed by a proline (also known as proline-directed phosphorylation) is a common post-translational signal inside the cell, especially in pathways of cell growth and proliferation. Any site phosphorylated by proline-directed kinases, such as CDKs, GSKs and MAPKs, has the potential to be a Pin1 binding site but it is unknown what makes a site specific for the attentions of Pin1.

Since its discovery, more than fifty Pin1 binding proteins covering diverse cellular processes have been identified (*8, 9*). While not all Pin1 interacting proteins have had their binding site(s) defined, those that have (see Chapter 4 for a list) show that the most commonly shared characteristic of Pin1 binding sites, aside from the pS/T-P motif, is their general placement in unstructured regions of the protein (*92*). This is not surprising as phosphorylation often takes place in unstructured regions. Also, the isomerization of a peptide bond in the middle of a folded domain will likely result in great structural and domain rearrangement of the whole protein and would therefore be avoided.

Other commonalities between Pin1 sites are difficult to determine. In this respect, they lack any other sequence features and can contain either a single site, such as in

Wee1, Cyclin D1 and APP, or may contain multiple sites such as the C-terminal tail of RNA polymerase II, which has 52 repeats of the Pin1 binding motif YpSPTpSPS (*34, 44, 55, 59, 93*). It has been noted that some multisite Pin1 interacting proteins, including Cdc25C, Tau, NHERF-1 and $p54^{nrb}$/NonO, have their Pin1 binding sites separated by 18-22 amino acids (*94*). It has been suggested that this may allow some kind of cooperativity between binding sites perhaps through a mode of binding that involves both the WW and PPIase domains of Pin1 (*62, 94*).

b) Possible modes of binding

That Pin1 has two domains capable of binding the same phosphomotif complicates the mechanism responsible for its binding to target proteins considerably. Although three possible binding mechanisms have been proposed for full-length Pin1, the simultaneous model, the sequential model and the catalysis first model (shown in Figure 1.4), no one model has had complete success in integrating what is known about Pin1 binding *in vitro* (discussed in section 1.4) with what is known about Pin1 binding sites and Pin1 function *in vivo*. Each model is explained in more detail below.

One possible mechanism, termed the simultaneous model (Figure 1.4A), has the higher affinity WW domain targeting one site on the protein which then increases the local concentration of the PPIase domain and allows it to bind and isomerize one or more nearby phosphosites (*78, 85, 95*). For this mechanism, the sites need not be close in primary sequence of course, or even on the same protein. If they were on the same protein it might explain why some Pin1 binding proteins prefer certain spacing between their sites. This model predicts that Pin1 could potentially isomerize many more sites than the few to which the WW domain can bind (*95*), although this is difficult to determine because of the problems of detecting isomerization *in vivo*. It does not explain,

however, why many Pin1 interacting proteins have a single site, especially in the case of Che1, a transcriptional activator that, after genotoxic stress, is degraded by a Pin1-dependent mechanism and which has only one S/T-P site in its sequence (*96*). It is always possible that these single site proteins may be involved in a multiprotein complex where another protein is actually the target of Pin1 isomerization. There is, however, currently no support for this mechanism in the literature.

In the sequential model (Figure 1.4B), the WW domain first binds a phosphomotif and then passes off that same site to the PPIase domain for isomerization (*91, 95*). This would leave the WW domain free to bind a neighbouring phosphosite effectively allowing Pin1 to "walk along" multiple sites in a target protein. This model would explain the spacing between multiple sites in Pin1 targets, but still allow for Pin1 isomerization of targets with a single site. However, how the lower affinity PPIase domain displaces the binding of the WW domain in this model is not known. Another potential complication arises from the ability of the WW domain to mainly bind sites that are in the *trans* conformation. This would imply that when passed off to the isomerase domain, catalysis occurs in the *trans* to *cis* direction, which conflicts with observations that Pin1 enhances the actions of the *trans*-specific phosphatase PP2A to dephosphorylate Pin1 sites in, for example, Cdc25C and Tau (*63, 78*).

The conflicts of the sequential model would be resolved in the catalysis first model originally proposed by Winjens *et. al* (2001) (Figure 1.4C) which states that any Pin1 site in the *trans* form would likely be bound by the WW domain. This leaves any available *cis* Pin1 sites for isomerization first by the PPIase domain. Once in the *trans* form, these sites would then be bound by the adjoining WW domain. This mechanism hinges on the ability of the low affinity PPIase domain to isomerize substrates without

prior targeting by the WW. Although the PPIase domain is able to work alone in yeast (*63*), it must be overexpressed, suggesting the WW domain is needed for normal Pin1 function. This mechanism would also suggest that Pin1 works at near stoichiometric levels in the cell, with one molecule of Pin1 for every binding site available. This would seem unlikely given the number of Pin1 targets during certain phases of the cell cycle, but Pin1 protein levels are subject to their own cell cycle-dependent and cell cycle-independent regulation (*49, 58, 97, 98*), and whether Pin1 acts on targets in stoichiometric or substoichiometric amounts is still an open question (*92, 99*).

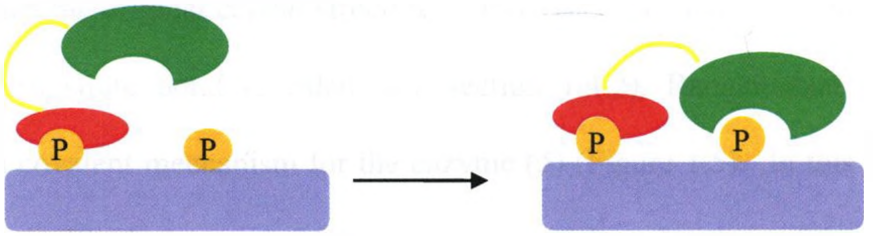
In general, each of these models has its own advantages and disadvantages and it is possible that the mode of binding of Pin1 may be substrate and/or organism specific.

1.5.2 Pin1 as an isomerase

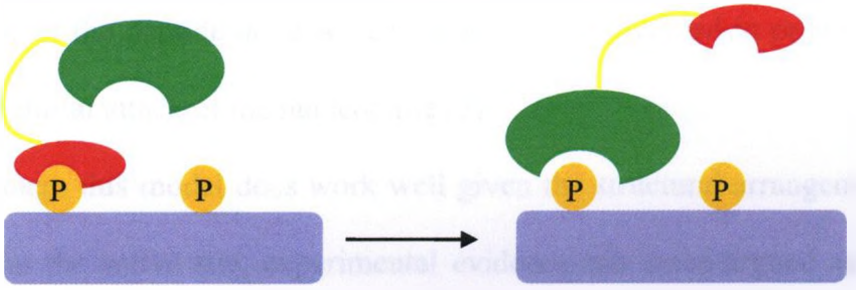
The ability of parvulins to catalyse *cis/trans* isomerization *in vitro* has been known since 1994 (*22*), and it has been confirmed many times in the literature that the PPIase domain of Pin1 is similarly capable of isomer conversion on small, often phosphorylated peptides (*5, 14, 21*). Unfortunately, even with structures of Pin1 and other parvulins available, the catalytic mechanism for these enzymes remains unknown. In fact, although structures of the other two families of PPIases, CyPs and FKBPs, have been solved, their mechanisms of isomerization remain similarly unclear (*13, 82*). While exact mechanisms are unknown, studies of PPIases to date have been most successful at eliminating mechanisms enabling minor refinements to probable catalytic processes. As described below, evidence suggests that Pin1 uses a non-covalent mechanism for catalysis. Given the chemical environment of the active site of Pin1 and other parvulins, isomerization may proceed through a bond distortion mechanism.

Figure 1.4- Mechanisms of Pin1 binding. A) The simultaneous model: the WW domain (shown in red) binds to a phosphosite on a protein increasing the local concentration of the PPIase domain (shown in green) which then acts on a proximal site on the same or a neighbouring protein. B) The sequential model: the WW targets a phosphosite allowing the PPIase domain to follow and act on the same site. C) The catalysis first model: the WW domain binds to all of the *trans* form of the phosphosite. The *cis* form are acted upon by the PPIase domain turning them into binding sites for the higher affinity WW domain.

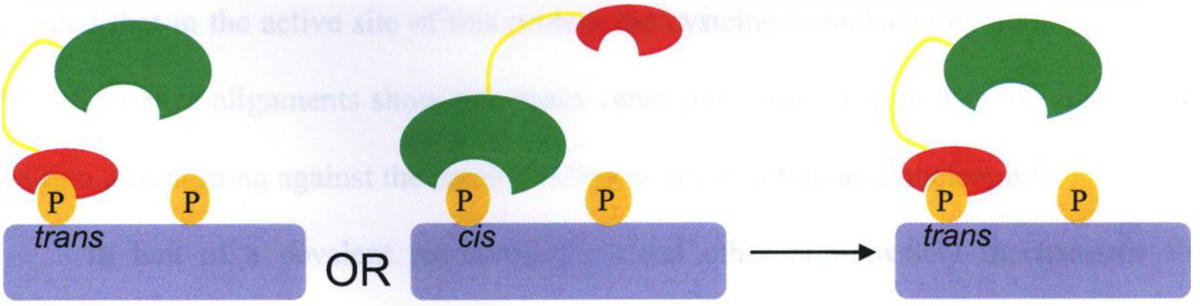
A) Simultaneous model



B) Sequential model



C) Catalysis first model



a) Evidence for a non-covalent mechanism

When the original crystal structure of Pin1 was solved and the placement of C113 above the substrate bond revealed (see section 1.4.2), Ranganathan and co-workers proposed a covalent mechanism for the enzyme (*5*) (Figure 1.5). In this mechanism, the nucleophilic sulfhydryl group of C113 attacks the carbonyl carbon of the peptide bond in the substrate. This results in a covalent enzyme-substrate intermediate with tetrahedral structure around the carbon so that when this intermediate collapses, it can form either the *cis* or *trans* isomer of the peptide bond. The two histidines, H59 and H157 which flank the underside of the peptide bond were proposed to be involved in proton redistribution, allowing the initial attack of the nucleophile (*5*).

Although this model does work well given the structural arrangement of this trio of residues in the active site, experimental evidence has since argued against this as a mechanism for Pin1 isomerization. First, in the *Arabidopsis thaliana* Pin1 homologue, NMR showed that the cysteine residue in the active site, C70, showed little chemical shift change upon ligand binding (*26*). Also, the structure of another human parvulin, Par14 revealed that in the active site of this protein the cysteine is replaced by an aspartic acid (*84*). Sequence alignments show that many other parvulins have an aspartic acid in this position also arguing against the residue in this position acting as a nucleophile.

In lieu of a covalent mechanism, several other non-covalent mechanisms for isomerization have been proposed including transition state binding where the enzyme binds to a more twisted state of the prolyl bond. However, recent structures of Pin1 bound to peptide inhibitors show that they can bind with angles near the *cis* (ω= -19°) and *trans* (ω= 183°) forms of the peptide suggesting this is not the most likely mechanism

(*69*). Another possible mechanism is *bond distortion* where the chemical environment of the enzyme reduces the partial double bond character normally found in amide bonds due to resonance (*10, 82*). The latter would generally be accomplished by the enzyme inducing more favourable interactions with the apolar form of the amide bond by either: 1) creating a more hydrophobic environment around the peptide bond; 2) creating an unfavourable positive environment around the amide nitrogen perhaps through donation of a hydrogen or 3) creating an unfavourable negative environment around the carbonyl oxygen. Work studying non-enzymatic peptide isomerization has shown that the barrier to rotation is mainly due to the hydrogen bonds formed to the peptide bond oxygen (*100*) and one way that the active site environment of Pin1 may work to decrease these hydrogen bonds is shown in Figure 1.5. In this model, C113 would be kept in a more negative state through hydrogen bonds with the rest of the active site and would not be able to present a positive hydrogen to the carbonyl oxygen. This would encourage the single bond form of the peptide bond allowing for rotation about the bond in a non-covalent manner. This model would also explain why an acidic residue, such as D, could be substituted for C in some parvulins as it would also present a negative charge to the carbonyl carbon.

b) Hints from other members of the PPIase family

Although CyPs and FKBPs vary in sequence, and in the case of Cyclophilins, in structure, from Pin1, it has been suggested that the three groups of PPIases may have similar mechanisms of catalysis (*82*). This is mainly due to the shared structural feature of the proline binding pocket which could potentially be used as an anchor in catalysis while the more flexible N-terminus is allowed to rotate around the peptide bond. For

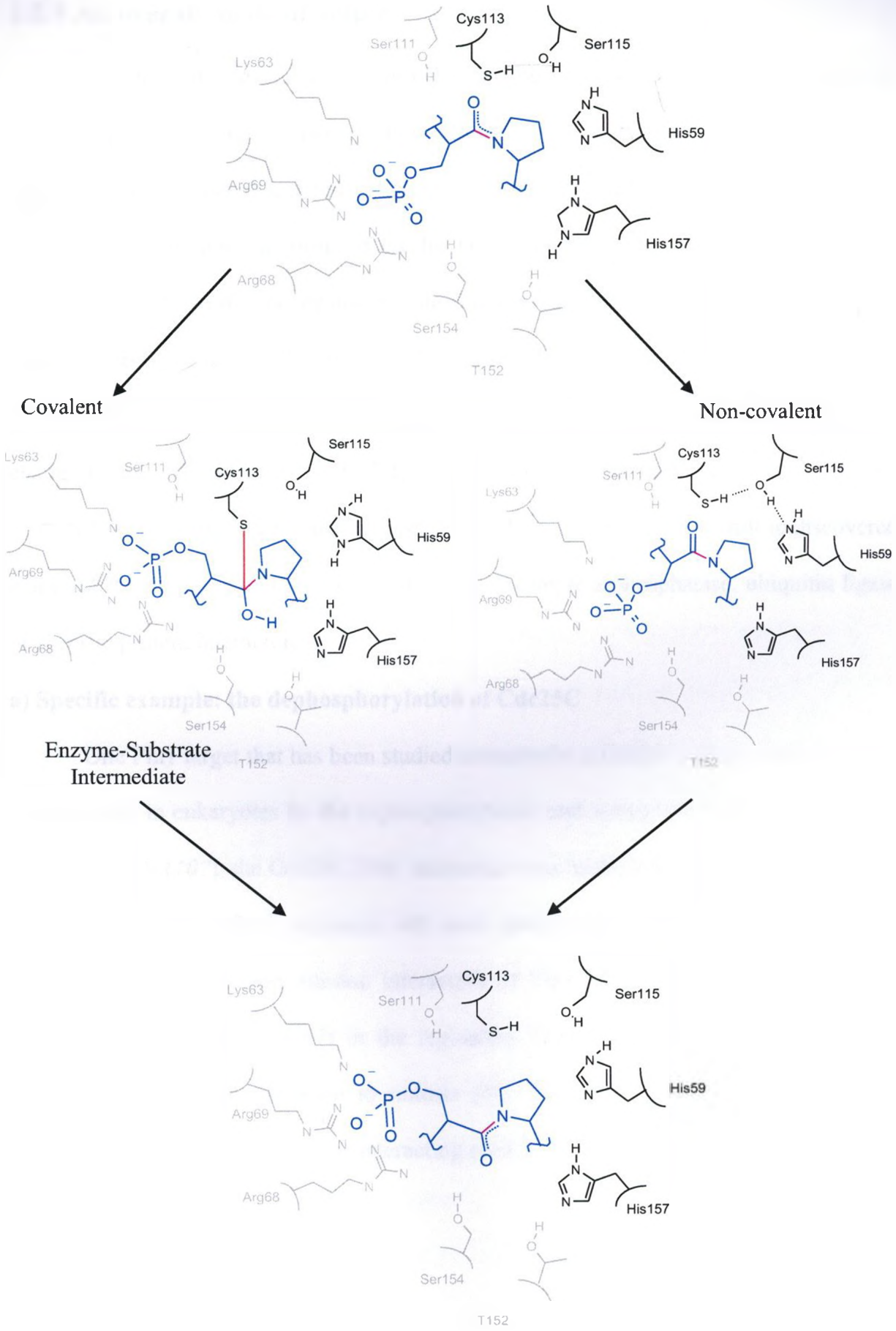
Pin1 at least, NMR studies have shown that this is true (*90*), and it is certainly possible that all PPIase catalytic mechanisms have common elements.

For CyP, mutagenesis studies have ruled out the possibility of cysteines acting as nucleophiles for catalysis (*101*). Instead, other non-covalent mechanisms have been considered. Work with synthesized potential transition state analogs suggests that the main mechanism is not through transition state binding (*102*). Instead, a possible mechanism may involve an arginine residue in the active site of CyP which may hydrogen bond to the amide nitrogen thereby reducing the partial bond character of the prolyl bond (*103, 104*). However, this mechanism is still in dispute (*13, 103*).

Determining the mechanism for FKBPs is more of a challenge as there hasn't yet been a structure solved with a potential substrate bound in the active site and even the prolyl binding pocket cannot be precisely determined (*82*). However, studies that have mutated potential nucleophiles near the active site have concluded that the mechanism used by FKBP, like that of other PPIases, is non-covalent (*105*). Although the precise mechanisms remain to be elucidated, molecular dynamics studies have suggested that a combination of the mechanisms listed above may be used (*106*).

It is interesting that all three groups of PPIases appear to use non-covalent mechanisms in catalysis. It may be that catalysis by PPIases involves more than one mechanism to reduce the activation energy of the reaction and that each group has its own unique combination. However, given the possibility that all PPIases may work similarly, the elucidation of a mechanism for one group of PPIases may mark a step forward in determining that of the others.

Figure 1.5- Covalent and non-covalent catalytic mechanisms for isomerization based on Pin1 active site structure. In covalent catalysis (shown on the left), C113 acts as a nucleophile and attacks the carbonyl carbon of the peptide bond, forming an enzyme-substrate intermediate which can then collapse to either the *cis* or *trans* isomer. In this model, H59 and H157 are involved in proton exchange. One example of non-covalent catalysis is shown on the right where unique hydrogen bonding in the PPIase active site allows C113 to present a partial negative charge to the carbonyl oxygen. This decreases the double bond character of the peptide bond allowing rotation. Diagram drawn using ISIS.


Cys113
Ser115
Ser111
Lys63
His59
Arg69
His157
Arg68
Ser154
T152
Covalent
Non-covalent
Enzyme-Substrate
Intermediate

1.5.3 An overall mode of action

The general role of Pin1 in cells that has been proposed is that it represents an extra regulatory step between two forms of a target: for example the phosphorylated/dephosphorylated forms of Cdc25C or Tau, the ubiquinated/deubiquinated forms of Cyclin D1, or nuclear/cytoplasmic forms of c-Jun. Whether Pin1 performs its regulatory role through catalysis or simple binding is what remains to be determined. In the case of catalysis, the conversion between *cis* and *trans* catalyzed by Pin1 would render one isomer pool more or less functional because of a change in its catalytic activity, stability, localization or phosphorylation status. If Pin1 acted through binding then it would work by facilitating interaction in still undiscovered manner between the target and its regulator, for example a phosphatase, ubiquitin ligase or specific protein interactor.

a) Specific example: the dephosphorylation of Cdc25C

One Pin1 target that has been studied extensively is Cdc25C. Due to its control of mitotic entry in eukaryotes by the dephosphorylation and activation of the mitotic kinase Cdc2/Cyclin B (*107*), the Cdc25C-Pin1 interaction was initially investigated as a possible mechanism for how Pin1 regulated cell cycle progression. Early studies found that Cdc25C was one of many mitotic interactors of Pin1 (*7*). Binding was due to two threonine residues (48 and 67) in the regulatory N-terminus of Cdc25C which were phosphorylated in the transition to mitosis (*6*). Phosphorylation of these and other residues allows dissociation of the interacting protein 14-3-3 and translocation of Cdc25C to the nucleus (*107*).

Pin1 is thought to act in this pathway in two ways. First, Pin1 causes changes in Cdc25C phosphatase activity *in vitro* either activating or inhibiting it depending on the phosphorylation status of the protein (*99*). Alternatively, Pin1 has also been shown to enhance the dephosphorylation of Cdc25C by the *trans*-specific phosphatase PP2A (*63*) and Pin1 depletion in *Xenopus* extracts causes Cdc25 hyperphosphorylation (*108*).

On a molecular level, Cdc25C is one of the best studied Pin1 targets besides Tau and the Pol II CTD and it arguably provides the best evidence that Pin1 can act as an isomerase on full-length proteins (*99*). Structural studies utilizing a peptide based on the T48 sequence of *Xenopus* Cdc25 showed that it could exist up to 10% in the *cis* form, making it a possible target for isomerization (*78*). However, the WW domain alone was found to bind the *trans* form of this peptide (*108*) while the PPIase domain alone could not bind this peptide at detectable levels (*91*). Still, a T48 peptide was found to be efficiently dephosphorylated in the presence of Pin1, but not in the presence of Pin1 active site mutants, suggesting that full-length Pin1 may be able to act catalytically on this substrate (*63*). Furthermore, one particularly thorough study by Stukenberg and Kirschner (2001) found that substoichiometric levels of Pin1 could affect a conformational change on the full-length Cdc25C protein *in vitro* detected using proteolysis and differential reactivity to a phospho-specific antibody (*99*). This study provided excellent evidence that Pin1 can catalyze isomerization on a full-length substrate and suggested that this may enable the dephosphorylation of the Pin1-generated *trans* form of Cdc25C by PP2A. But evidence is still lacking to show that Pin1 acts on *cis* or *trans* isomers *in vivo*. Instead, Pin1 may simply facilitate Cdc25C dephosphorylation by PP2A through an as yet undiscovered binding mechanism (*92*).

1.6 Objectives

Evidence that Pin1 plays an important regulatory role in cells and may represent a potential therapeutic target because of its involvement in disease has been presented in this introductory chapter and extensively in literature reviews. There have also been numerous structural studies which indicate that Pin1 can bind and catalyze phospho-specific motifs *in vitro*. Questions still arise, however, as to how Pin1 performs its role in cells. It is the goal of this thesis to bridge the gap between previous structural and functional studies to better determine the molecular mechanisms of Pin1 action *in vivo*. Accordingly, we hypothesize that Pin1 acts as an extra regulatory step in signalling pathways by first binding to phospho-specific targets with its WW domain and then catalyzing the *cis* to *trans* isomerization of phosphomotifs in those same proteins using its PPIase domain.

To begin investigating this hypothesis, we first designed a study to look for functionally important residues in Pin1. Many of the mutations used in previous Pin1 studies were chosen based upon structural information, but an unbiased evaluation of residues important in Pin1 function had not been performed. Based upon the results of a unigenic evolution study performed previously in the lab (*109*), where the human Pin1 gene was randomly mutagenized and functional mutations selected for based upon their ability to support viability in yeast, key regions of Pin1 were found that were important for function. Specific residues in these regions were chosen for further investigation based upon either their conservation in the unigenic (and natural) evolution or because their mutations were found in the unigenic evolution analysis but were unexpected because of results from other structural or functional studies. These tests led to the

definition of important structural, functional and catalytic roles for a number of residues in Pin1, most notably C113 and H59 and H157, as well as refinement for the catalytic model of Pin1. These results are discussed in Chapters 2 and 3.

Secondly, we examined the binding of Pin1 to its interacting proteins. Using mutations with known defects in PPIase domain binding, we investigated the effect of these mutations on Pin1 interactions *in vitro*. Interestingly, we found that there are two classes of Pin1 interacting proteins, differing likely in their interaction with the Pin1 PPIase domain. Furthermore, a review of known Pin1 sites in the literature suggested that binding of the domains of Pin1 may be determined more by the structures of sites rather than the sequence. We also tested the effect of single and multiple sites on Pin1 binding by mutating residues in the well-defined interacting protein Cdc25C. The results of this study are discussed in Chapter 4.

In summary, our studies have integrated both structural and functional information to more precisely determine the molecular mechanisms of Pin1 action in cells. Our results indicate that Pin1 in unique amongst PPIases in its ability to bind and catalyze the isomerization of phosphorylated motifs likely enabling its important cellular role.

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Chapter 2: Investigation of important functional residues in Pin1[1]

2.1 Introduction

Originally characterized as a mitotic regulator (*1*), the peptidyl-prolyl isomerase (PPIase) Pin1 has since been implicated in various aspects of cell signalling due to its ability to bind and isomerize sequences containing a phosphorylated serine or threonine followed by a proline (pS/T-P motifs) (*2-5*). Indeed, Pin1 has been involved in such diverse signalling processes as transcription, DNA damage, apoptosis, immune responses and neuronal survival (*6-24*). Furthermore, alterations of Pin1 have been linked to diseases such as Alzheimer's Disease and cancer (*25-28*). Based on its diverse cellular roles, Pin1 represents a good candidate for further study as a regulator of signalling processes.

Pin1 is a member of the parvulin family of PPIases and consists of two domains (*1, 5*). The N-terminal WW domain is responsible for high affinity binding to phosphorylated targets (*4, 29*). Like other WW domains, it is a three-stranded β-sheet that contains two conserved tryptophan residues separated by 20-22 amino acids and is specific for proline-rich sequences (*5, 29, 30*). The C-terminal PPIase domain shares sequence similarity with other parvulins, but unlike the bacterial PPIases, the Pin1 catalytic domain specifically isomerizes peptidyl-prolyl bonds that are preceded by a phosphorylated serine or threonine (*5, 31*). This phosphospecificity is mainly attributed

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to the presence of a phospho-specific binding loop, which contains several basic amino acids, that is absent in non-phosphospecific parvulins (*5*).

Although several structures of Pin1 have been solved (*5, 29, 32, 33*), the study of Pin1 remains incomplete as it is not known how the enzyme works in cells. Such knowledge would also be essential for the design of any therapeutic intervention for Pin1. As a first step in investigating how Pin1 performs its cellular function, a previous study in the lab used unigenic evolution, a process where random mutations are introduced into a specific gene and functional mutations are selected for revealing those regions of the gene that are not necessary for function. The unigenic evolution was used previously to select for the Pin1 mutations that could functionally compensate for the loss of the Pin1 homologue, Ess1, in *Saccharomyces cerevisiae* (*34, 35*). Consequently, this strategy allowed for an unbiased approach to discovering regions and residues in Pin1 which were important for function whereas mutations determined in the past were mainly selected based on their position in Pin1 structures. This unigenic evolution study revealed four "hypomutable" regions, those which had fewer observed mutations than expected, which we have examined further here by generating site-directed mutations of specific residues in these regions of Pin1. These mutants were tested for both their function in yeast and their isomerase activity *in vitro*.

2.2 Materials and Methods

Plasmid Construction

For expression of Pin1 mutants in the yeast plasmid shuffling assay, site-directed mutagenesis was performed on pY204-Pin1, a *LEU2*-containing centromeric plasmid with a truncated *DED1* promoter used previously in the unigenic evolution analysis (*34*). The mutants Y23F, K63A, R68A, and R69A were generated by Norclone (London, Ontario, Canada). The R68/69A and Y23A mutants were made from mutants constructed previously by Messenger et al. (*36*) by using the Pin forward (PinF) primer: 5′-ATA AGA ATG CGG CCG CCATGG CGG ACG AGG AGA-3′ and Pin reverse (PinR) primer: 5′-GGA ATT CTC AGT CAC GAT GAA TAA GCT TCA-3′ to introduce NotI and EcoRI sites using the polymerase chain reaction (PCR). Amplified products were subcloned into pY204 using NotI and EcoRI. Single-site mutants R69W and H59Q were obtained during unigenic evolution and used in further studies. Other mutants were synthesized using PCR-mediated site-directed mutagenesis and the following primers: C113N: 5′-CAG TTC AGC GAC AAC AGC TCA GCC AAG-3′(forward) and 5′-CTT GGC TGA GCT GTT GTC GCT GAA CTG-3′ (reverse); C113S: 5′-CAG TTC AGC GAC AGC AGC TCA GCC AAG-3′ (forward) and 5′-CTT GGC TGA GCT GCT GTC GCTGAACTG-3′ (reverse); H59L: 5′-GTC CGC TGC TCG CTC CTG CTG GTG AAG-3′ (forward) and 5′-CTT CAC CAG CAG GAG CGA GCA GCG GAC-3′ (reverse); H157L: 5′-GGA ATT CTC TCA CTC AGT GCG GAG GAT GAT GAG GAT GCC GGA-3′ (reverse); H157N: 5′- CCA AGC TTC ACT CAG TGC GGAGGATGATGT TGA TGC CGG AAT C-3′ (reverse) along with the PinF and PinR primers to generate products that could subsequently be subcloned into NotI/EcoRI -digested pY204. The C113D mutant was

made using the oligos: 5′-CAG TTC AGC GAC GAC AGC TCA GCC AAG-3′ (forward) and 5′-CTT GGC TGA GCT GTC GTC GCT GAA CTG-3′ (reverse) with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's instructions. For bacterial expression, a pPro-ExHta-Pin1 vector with a His_6 N-terminal tag was used. Mutants to be expressed and purified were either subcloned in to this vector from pY204 (K63A, R68/69A, C113S) or made by site-directed mutagenesis using the same primers and methods listed above (C113D).

Yeast transformation and plasmid shuffling assay

The yeast strain YKH100 (genotype, MAT*a ura3-1 leu2-3, 112 trp1-1 can1-100 ade2-1 his3-11*, 15[phi+] *ess1Δ::TRP1*) which maintains growth by expressing wild-type human Pin1 on the *URA3*-containing plasmid YCp88Pin1 (*34*), was transformed with pY204-Pin1 wild-type and mutant constructs. Transformants which grew on synthetic complete agar plates lacking tryptophan and leucine (SC-W-L) were then grown overnight in liquid media. Cultures were equalized for cell density and 10μl aliquots of 1/10, 1/100, 1/1000, 1/10 000 and 1/100 000 serial dultions were spotted on minimal agar with 1mg/mL of 5-fluoroorotic acid (Toronto Research Chemicals, Inc.) and allowed to grow for three to five days.

Western blotting

For Western blotting, wild-type Pin1, C113D, C113S and C113N mutants were expressed with an N-terminal triple FLAG tag. Yeast strains were grown in 5 ml of selective media (-W, -L, -U) with the exception of the YKH100 strain that was grown in YPAD, and harvested by centrifugation. Yeast were then lysed in 100 μl of buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, and 1 mM EDTA similar to that

described by Gill et al. (*37*), except that samples were vortexed ten times for 30 s each. Supernatants were obtained by centrifuging at 13,000 g for 5 min. Protein concentrations were determined using the Bradford protein assay (BioRad) and 125 μg of each sample was separated by SDS–PAGE and transferred to polyvinyl difluoride membrane. Blots were probed using anti-FLAG (M2) antibody (Sigma) and immune-complexes detected using enhanced chemiluminescence (Super-Signal, West Pico from Pierce) according to the manufacturer's specifications.

Bacterial Pin1 purification and PPIase assays

For expression of His_6-Pin1 proteins, overnight cultures were diluted 1/1000 in LB supplemented with 100 μg/mL ampicillin (Roche) and allowed to grow at 37 °C until OD_{600} reached 0.6. Expression was induced with 0.6 mM isopropylthio-α-D-β-galactoside (Roche) at 37 °C for 3-5 hours before bacteria were pelleted. Bacteria were lysed by sonication and cell debris spun out using centrifugation. His-tagged proteins were then purified on HisTrapHP or nickel-saturated chelating sepharose (Amersham Biosciences) and eluted with 100-250 mM imidazole. Proteins were then run on SDS-PAGE and stained with Coomassie Blue to assess purity before dialysis into 50 mM sodium phosphate (pH 7.7), 150 mM NaCl, 10% (v/v) glycerol for storage at -20 °C.

PPIase activity measurements were determined using a spectrophotometric assay similar to Kofron *et al.* (*38*). Assay readings were carried out at 0 °C in a Cary-100 spectrophotometer. As substrate either Suc-AEPF-*p*NA or WFYpSPR-*p*NA (Pintide) dissolved in 0.3 M LiCl/TFE was added to 2 mL of assay buffer (50 mMM Hepes, pH 7.5, 100 mM NaCl, 5 mM NaN_3). Either chymotrypsin (for the Suc-AEPF-*p*NA substrate) or trypsin (for the WFYpSPR-*p*NA substrate) (both from Sigma) were then

added to a final concentration of ~1.25 mg/mL. A reading of the rate of chemical isomerization was measured for 30 s before the addition of Pin1 protein. Measurement of the rate of enzyme plus chemical isomerization was then taken. The chemical isomerization rate was subtracted from the final rate. For AEPF measurements, data parameters were calculated using a non-linear least-squares fit to the Michaelis-Menten equation in GraphPad Prism. For WFYpSR (Pintide) data, only low concentrations of peptide were used and k_{cat}/K_M was calculated based on the slope of a rate versus substrate concentration plot.

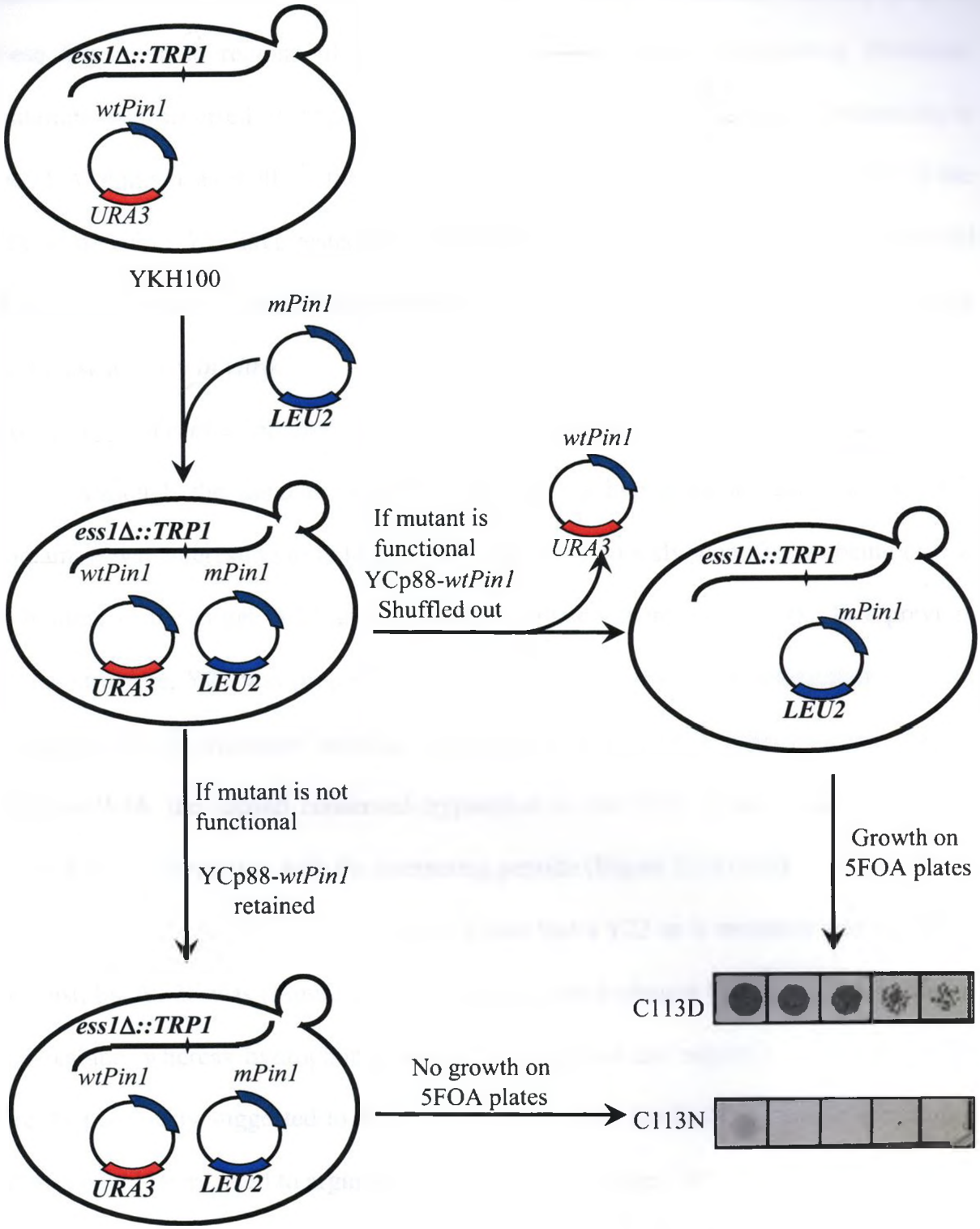
2.3 Results

Design of an assay in yeast to screen for functionality of Pin1 mutations

In *S. cerevisiae*, the Pin1 homologue, Ess1, is essential for cell viability (*39*). The loss of Ess1 can be complemented, however, by the expression of human Pin1 (*1*). A plasmid shuffling assay was designed around this principle to allow for large-scale screening of functional mutations of human Pin1 in yeast (Figure 2.1). In this strategy, yeast containing a knockout of the ess1 gene are complemented by human wild-type Pin1 expressed on a *URA3*-containing plasmid. This strain (called YKH100) is then transformed with a mutant Pin1 allele on a *LEU2*-containing plasmid. Yeast containing both wild-type Pin1 and the mutation to be tested are then grown in non-selective media and plated on 5-fluroorotic acid (5FOA) which is toxic to yeast containing the *URA3* allele. In cases where the Pin1 mutation is functional, the *URA3*-containing wild-type Pin1 plasmid can be shuffled out of the yeast during unselective growth and yeast containing only the *LEU2*-Pin1 plasmid will grow on 5FOA. Conversely, if the Pin1 mutation is not functional, the *URA3*-containing wild-type Pin1 plasmid is retained for growth and these yeast will not grow on plates containing 5FOA.

This plasmid shuffling assay was used previously in the lab in a unigenic evolution study whereby randomly generated pools of Pin1 mutations were tested for their ability to complement the ess1 deletion in yeast (*34*). Functional mutations were gathered and sequenced and results revealed four regions of Pin1 that were "hypomutable" where functional mutations were less likely to be found (Figure 2.2A). Region A (residues 29-33) is in the WW domain while regions B, C and D (56-70, 107-120, 139-158, respectively) are in the PPIase domain around the catalytic pocket. To

Figure 2.1- Plasmid shuffling assay for identification of functional Pin1 alleles. The YKH100 strain of *S. cerevisiae* contains a deletion of the ess1 gene which is complemented by the expression of human Pin1 on a *URA3* plasmid. This strain is then transformed with the Pin1 mutation to be tested on a *LEU2*-containing plasmid. Transformants containing both plasmids are then grown in unselective media where one of two things can happen. If the Pin1 mutation is functional (pathway to the *right*), then the *URA3* plasmid can be shuffled out and when plated on 5-fluoroorotic acid (5FOA), these yeast will grow (eg. C113D). If the Pin1 mutation is not functional (pathway on the *bottom*), then the *URA3* plasmid can be retained for growth, but this will be toxic for yeast grown on 5FOA and these yeast will not grow (eg. C113N).


ess1Δ::TRP1
wtPin1
URA3
YKH100
mPin1
LEU2
ess1Δ::TRP1
wtPin1
mPin1
URA3
LEU2
If mutant is functional
YCp88-wtPin1 Shuffled out
wtPin1
URA3
ess1Δ::TRP1
mPin1
LEU2
If mutant is not functional
YCp88-wtPin1 retained
Growth on 5FOA plates
ess1Δ::TRP1
wtPin1
mPin1
URA3
LEU2
No growth on 5FOA plates
C113D
C113N

extend this analysis, the goal of the study described here is to test key residues found in these hypomutable regions of Pin1 as well as to re-examine interesting functional mutations that occurred. Using site-directed mutagenesis, we have made substitutions in the WW domain as well as the phospho-specific binding loop and active site of the PPIase domain. We have tested these mutations for functionality in our yeast plasmid shuffling assay and for interesting mutations in the PPIase domain, have also tested their isomerase activity *in vitro*.

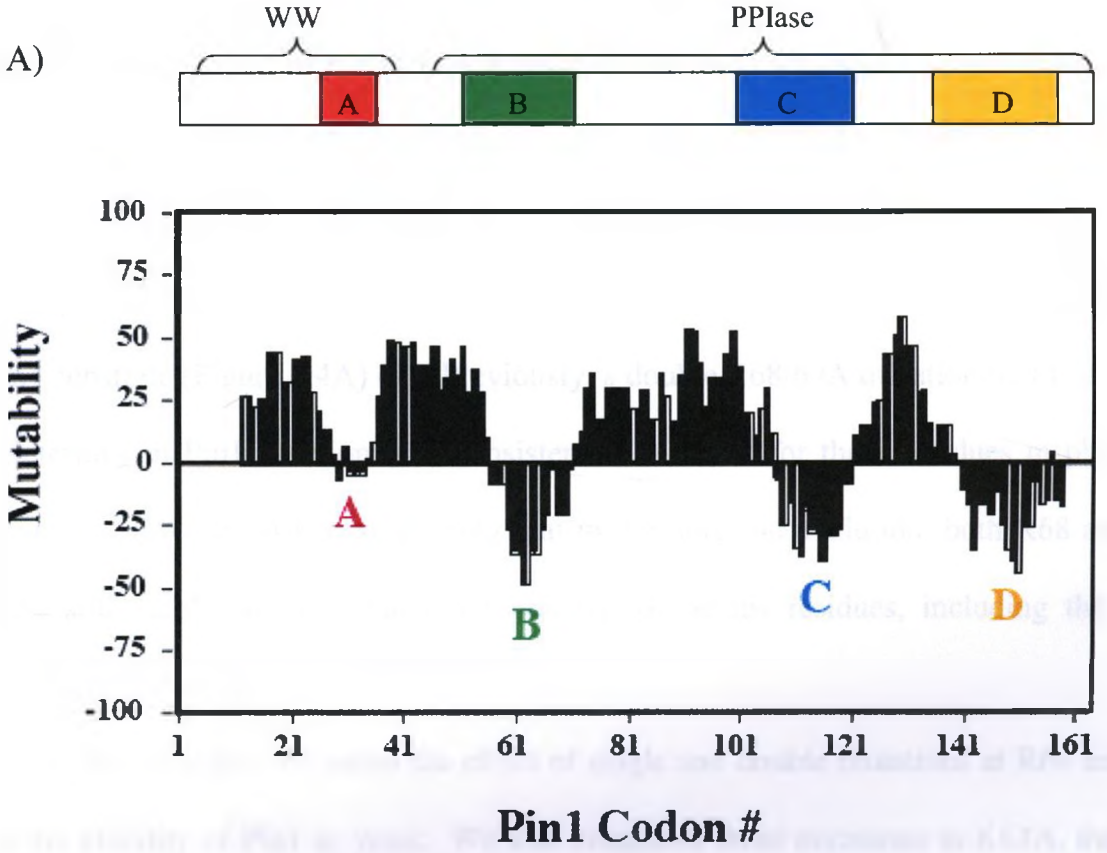
Examination of critical binding residues in the WW domain

Although the unigenic evolution did have a hypomutable region in the WW domain, it was interesting to note that two residues previously identified as being critical in binding to the target, Y23 and W34, were mutated in the study (*34*). In a previous crystal structure, Y23 was proposed to be involved in both stacking interactions with the target proline and hydrogen bonding to the phosphate moiety through a water molecule, whereas W34, the second conserved tryptophan in the WW domain, was involved in close stacking interaction with the interacting peptide (Figure 2.3A) (*29*).

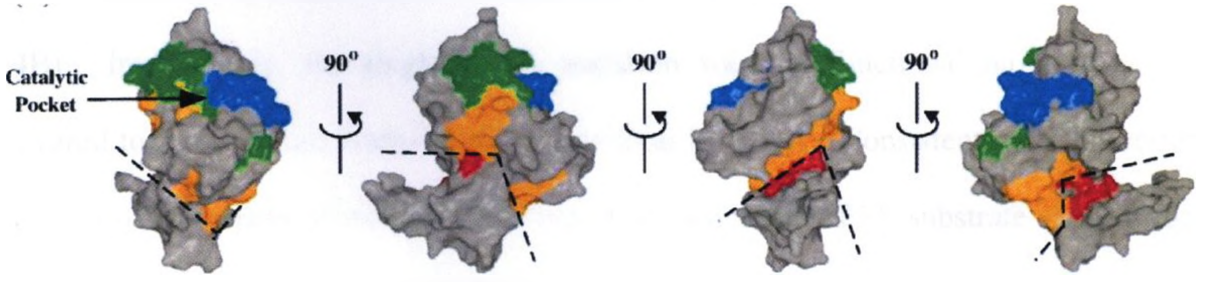
In our plasmid shuffling assay, we found that a Y23 to A mutation was not viable in yeast, but Y23F was (Figure 2.3B) suggesting that hydrogen bonding is not needed at this residue, whereas hydrophobic stacking interactions are required. Similarly, W34, already previously suggested to be essential for Pin1 on the basis of alanine substitution (*4, 40*), could be mutated to arginine and still retain function. The results shown in Figure 2.2 emphasize that while alanine scanning may indicate key functional residues, non-alanine mutations can also be useful in determining the types of interactions those residues require. In this respect, Y23 and W34 appear to be involved in the van der Waals interactions with the target pS/T-P motif in the WW domain.

Figure 2.2- Identification of hypomutable regions of Pin1 as determined in Behrsin *et. al (34).* A) The observed ratio of missense to total mutations (silent+missense) generated by unigenic evolution is compared with the expected ratio (determined by random clones) and the normalized mutability value for each codon is plotted on the x axis at the center of an 11 codon window for Pin1. The averaged ratio of missense to total mutations for each 11 codon region is given as a percentage of the maximal ratio possible. Maximal hypo-mutability (no missense mutations) is normalized to a value of −100, and maximal hyper-mutability (all missense mutations) is normalized to a value of +100. A value of 0 occurs when the observed and expected frequency of mutation are equal. Positive numbers (0–100%) along the y axis indicate percentage of maximal hyper-mutability, and negative numbers (0 to −100%) indicate percentage of maximal hypo-mutability. Four regions of negative mutability scores are indicated: A (codons 29–33), B (56–70), C (107–120), and D (139–158). B) Structural representation of hypo-mutable regions of Pin1. Conserved residues within each hypomutable region A (red), B (green), C (blue) and D (orange) from (a) (above) have been mapped to a van derWaals surface representation of human Pin1 (PDB: 1PIN). The approximate position of the boundary between the WW domain and the isomerase domain of Pin1 is highlighted. In each case, the WW domain is situated below the dotted line. Each of the illustrations has been rotated by 90° around a vertical axis as compared to its neighboring illustration.

A)


WW
PPIase
A
B
C
D
Mutability
100
75
50
25
0
-25
-50
-75
-100
1
21
41
61
81
101
121
141
161
A
B
C
D
Pin1 Codon #

B)


Catalytic
Pocket
90°
90°
90°

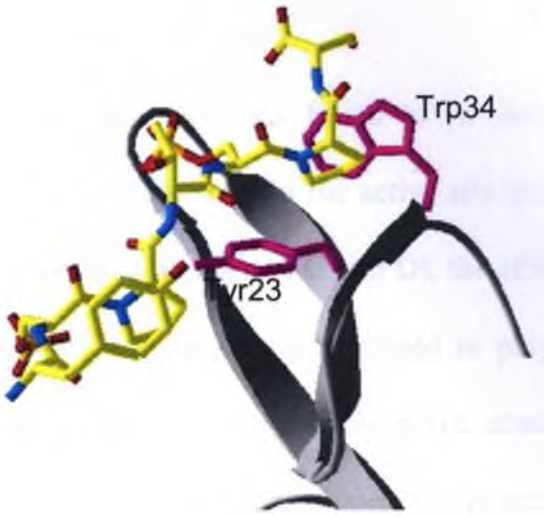
Mutation of residues involved in phosphate binding in the PPIase domain

In the unigenic evolution, most of hypomutable region B is part of the phospho-specific binding loop in the PPIase domain. This loop, which is thought to have some flexibility in Pin1 (*29, 41*), is not found in the bacterial parvulins (*42, 43*) which do not recognize a phosphate. The loop contains three basic residues, K63, R68 and R69, which were proposed to make electrostatic contacts with the negative phosphate moiety of the Pin1 substrate (Figure 2.4A) (*5*). Previously, a double R68/69A mutation was found to be deleterious in Pin1 function (*40*) consistent with a role for these residues in phosphate binding. However, it was interesting that in the unigenic evolution, both R68 and R69 were still viable when mutated individually to various residues, including the large, aromatic tryptophan.

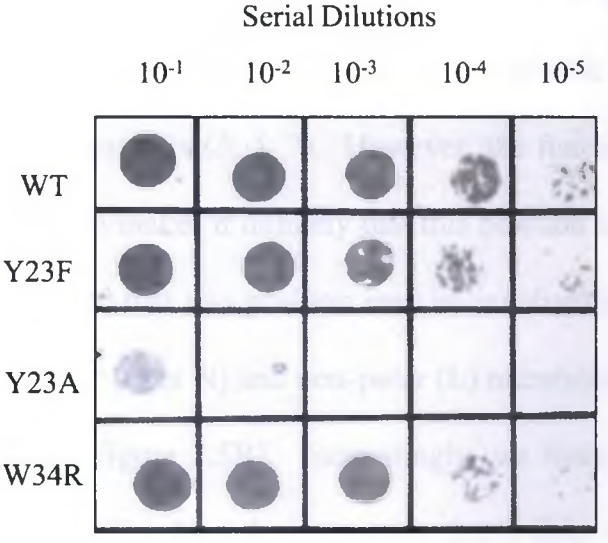
Accordingly, we tested the effect of single and double mutations at R68 and R69 on the viability of Pin1 in yeast. We also compared these mutations to K63A, the other basic residue in the phospho-specific binding loop, which was not found to be mutated in the unigenic evolution. In our plasmid shuffling assay, we found that R68 and R69 could be mutated singly to A and retain function, but a double mutation was deleterious (Figure 2.4B). Interestingly, the single K63A mutation was not functional on its own and appeared to have a more dramatic phenotype than R68/69A. Consistent with this result, the *in vitro* isomerase activity of these mutations using an AEPF substrate showed that while both the R68/69A and K63A mutations had less activity than wild-type Pin1, K63A had more of an effect than the double arginine mutation (Figure 2.4C). These mutations had no detectable activity using a phosphorylated WFYpSPR-*p*NA (Pintide) substrate (data not shown). Together, these results demonstrate that only one of the two arginines

Figure 2.3- Mutation of important binding residues in the WW domain. A) Ribbon diagram of the WW domain highlighting two aromatic residues, Y23 and W34 (shown in magenta), that have been implicated in target interactions. The bound phosphopeptide is based on the RNA polymerase II C-terminal domain (yellow carbon atoms; PDB: 1F8A). B) Yeast plasmid shuffling assay results. Site-directed mutagenesis was performed on both Y23 and W34. Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density and serial dilutions were spotted onto 5FOA plates to monitor growth.

A)


Trp34
Tyr23

B)


Serial Dilutions
10-1
10-2
10-3
10-4
10-5
WT
Y23F
Y23A
W34R

is required for function in the phosphate-binding loop and that K63 is the most important basic residue of the three.

Investigations of key residues in the active site

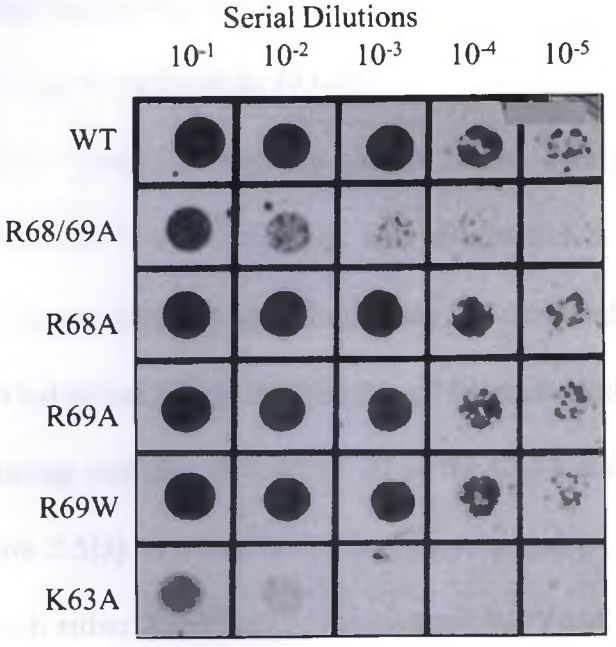
One of the most surprising mutations found to be functional in the random mutagenesis was H59Q. Although regions near the active site of Pin1 did show a lower frequency of mutation (ie. hypomutable regions C and D), the H59Q mutation was more unexpected because this residue was originally proposed to play a crucial role in the catalytic mechanism of Pin1. In this respect, a nucleophilic attack catalytic mechanism was proposed on the basis of the location of residues in the active site of the original structure of Pin1 (*5*). In this mechanism, a cysteine residue located above the peptidyl-prolyl bond, C113, attacks the carbonyl carbon (Figure 2.5A). H59 and its neighbour H157 were proposed to be involved in proton rearrangement in this model with H59 acting as a catalytic base. Initial mutation of H59 to hydrophobic residues did suggest that it could be important in catalysis (*3, 5, 7*). However, the functional H59Q mutation found in the unigenic evolution makes it unlikely that this position is acting as a catalytic base. Instead, we hypothesized that this position may be involved in hydrogen bonding. Therefore, we tested both polar (Q or N) and non-polar (L) mutations at H59 and H157 in our plasmid shuffling assay (Figure 2.5B). Interestingly, we found that position H157 could tolerate substitution of either N or L, suggesting that it does not require hydrogen bonding for function. H59, in contrast, could not tolerate mutation to L in the plasmid shuffling assay. Collectively, these results suggest that hydrogen bonding may be critical at the H59 position for Pin1 function.

The mutational data from H59 and H157 in Figure 2.5B are inconsistent with the original nucleophilic attack model for Pin1 catalysis. To further examine other

Figure 2.4- Investigation of basic residues in the PPIase phospho-specific binding loop. A) Structural diagram of the Pin1 PPIase domain with a bound sulfate molecule (shown in red; PDB: 1PIN). Basic residues in the phospho-specific binding loop are shown as green stick models. B) Yeast plasmid shuffling assay. Site-directed mutagenesis was performed on K63, R68 and R69. Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density and serial dilutions were spotted onto 5FOA plates to monitor growth. C) *In vitro* isomerase activity results. Mutant proteins were expressed and purified and used in the standard protease-coupled spectrophotometric assay with Suc-AEPF-*p*NA used as substrate.

A)

B)



C)

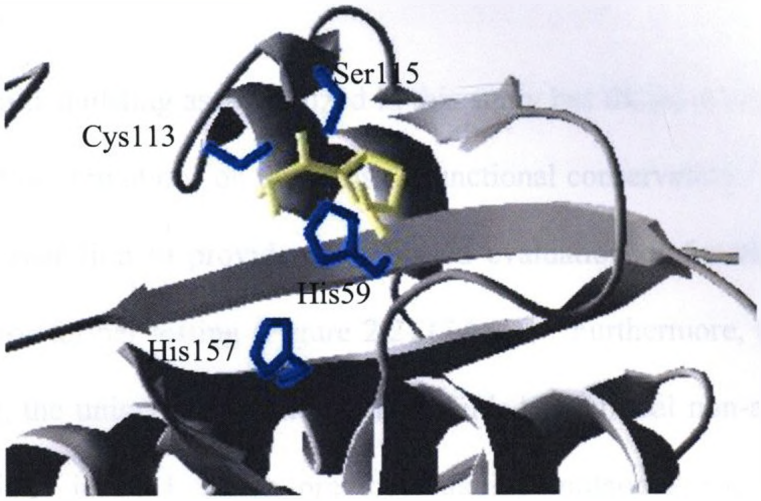
Protein	Suc-AEPF-*p*NA % WT activity
R68/69A	25
K63A	3.6

mechanisms for how Pin1 may isomerize its substrates, we looked at the other active site residue implicated in Pin1 catalysis, C113. This residue was not mutated in the unigenic evolution study and mutation of C113 to A or S had previously been found to render Pin1 non-functional in yeast with a corresponding decrease its catalytic activity (*40*). In the Pin1 structure, C113 sits at the top of the active site and points towards the carbonyl oxygen of the substrate. Its position in the active site puts it within hydrogen bonding distance of other polar residues such as H59 and S115. Furthermore, previous structural studies and multiple sequence alignment have shown that this residue can be replaced by a D in other parvulins suggesting that it is a negative charge that is more important at this position rather than a good nucleophile (*43-45*).

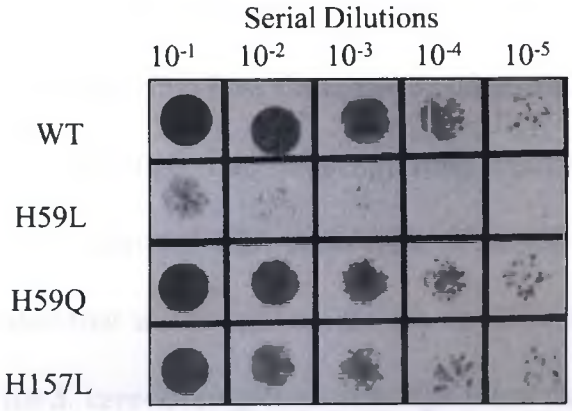
To test if Pin1 prefers a negative charge on its catalytic residue, we mutated position C113 to D, which is more negative, and to S, which is a better nucleophile than D, and tested them in our plasmid shuffling assay. Consistent with our prediction, we found that a C113D substitution was still functional in yeast while C113S was not (Figure 2.5C). Western blotting analysis shows that all of the C113 mutations were expressed at similar levels (Figure 2.5D). Furthermore, isomerase activity *in vitro* of the C113D and C113S mutations with either AEPF or phosphorylated WFYpSPR substrate showed lower activity for C113S consistent with yeast results. Collectively, Figure 2.5 suggests that Pin1 function requires a negative charge at C113 and that a charge at this position may in part be maintained by a hydrogen bond to H59.

Figure 2.5- Investigation of Pin1 active site residues. A) Structural diagram of the Pin1 PPIase domain with a bound Ala-Pro dipeptide (shown in light yellow; PDB: 1PIN). Key residues are shown as blue stick models. B) Yeast plasmid shuffling assay for the two conserved active site histidines. Site-directed mutagenesis was performed on H59 and H157. Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density and serial dilutions were spotted onto 5FOA plates to monitor growth. C) Yeast plasmid shuffling assay for the proposed catalytic cysteine. Site-directed mutagenesis was performed on C113. Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density and serial dilutions were spotted onto 5FOA plates to monitor growth. D) Expression of C113 mutations in yeast by Western blotting for FLAG. YKH100 does not contain FLAG-Pin1 and was used as a negative control. E) *In vitro* isomerase activity results. Proteins harbouring C113D and C113S mutations were expressed and purified and used in the standard protease-coupled spectrophotometric assay with Suc-AEPF-*p*NA and WFYpSPR-*p*NA (Pintide) used as substrate.

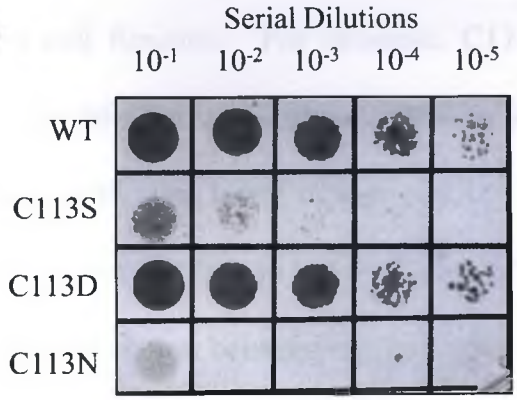
A)



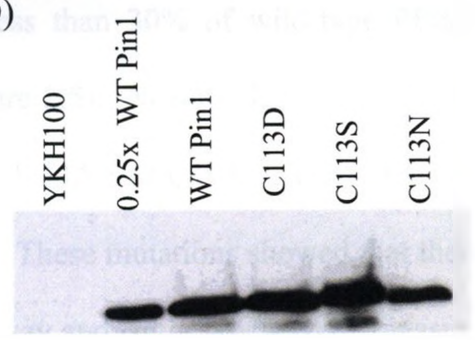
B)



C)



D)



E)

Protein	Suc-AEPF-*p*NA % WT activity	WFYpSPR-*p*NA % WT activity
C113D	17.7	28.6
C113S	2.0	1.2

2.4 Discussion

The plasmid shuffling assay utilized in this study has the advantage of assessing a large number of Pin1 mutations on the basis of functional conservation. This enabled the use of unigenic evolution to provide an unbiased evaluation of functionally important regions of Pin1 for further testing (Figure 2.2) (*34, 35*). Furthermore, unlike traditional alanine scanning, the unigenic evolution study yielded functional non-alanine mutations at several positions in Pin1 which previous alanine mutations had deemed critical. Testing of the non-alanine mutations Y23F and W34R in our yeast plasmid shuffling assay provided new insights into how these two residues may be working in WW domain binding. However, although our yeast plasmid shuffling assay was valuable in determining the functionality of Pin1 mutations, it should be noted that Gemmil *et. al* recently demonstrated that under optimal growth conditions, less than 400 molecules of Ess1 are required for *S. cerevisiae* growth although more than 200 000 molecules exist in the cell (*46*). This suggests that functional Pin1 mutations could vary in their abilities to perform cell function. For example, C113D had less than 30% of wild-type PPIase activity *in vitro* but was still functional in yeast (Figure 2.5). Nevertheless, a number of mutations with even lower PPIase activity, R68/69A, K63A and C113S were tested and did not support viability in yeast (Figure 2.4 and 2.5). These mutations showed that there is a good correlation between our plasmid shuffling assay and isomerase activity measure *in vitro* and suggest that there may be a threshold of PPIase activity which is needed for function in *S. cerevisiae*.

In examining residues in the phospho-specific binding loop, we found that K63 was the most critical basic amino acid needed for Pin1 function in yeast and for PPIase

activity (Figure 2.4). In contrast, it appears only one of R68 or R69 is needed for proper Pin1 function. In the original crystal structure of Pin1, all three of these residues were proposed to make electrostatic contacts with the phosphate moiety of the substrate (*5*). However, a more recent crystal structure with a peptide inhibitor bound in the active site showed that only K63 and R69 make close contact with the substrate phosphate (*32*). This is consistent with data by Rippman *et al.* who showed that while R to L substitutions at either 68 or 69 decrease isomerase activity compared with wild-type, the R69L mutations had a more dramatic effect (*15*). This suggests that R69 is the more important arginine in the Pin1 phosphate binding loop, but our data would also imply that in the absence of R69, R68 can function in a similar binding role. Comparison of the sequences of this phosphate binding loop in Pin1-like enzyme from other organisms shows that *both* arginines are generally conserved. It is possible that this loop, which is flexible to allow for rotation of the N-terminus of the substrate during isomerization (*29, 41*), may retain both arginines to allow for optimal, although not essential, continual contact to the phosphate throughout catalytic rotation.

Our analysis of residues in the active site of Pin1 demonstrated that catalysis does not proceed by the original nucleophlic attack mechanism proposed (Figure 2.5). We showed that in the plasmid shuffling assay, H59 can be mutated to Q and still support viability, indicating that it does not act as a catalytic base as originally proposed. As well, at position C113, D was a better replacement than S, suggesting that it is a negative charge at C113 which may be important. This is consistent with this position being changed to a D in other parvulins. A non-covalent mechanism for Pin1 which would require a negative charge is shown in Figure 2.6. In general, this mechanism works by lowering the energy barrier for peptide bond rotation through stabilization of the more

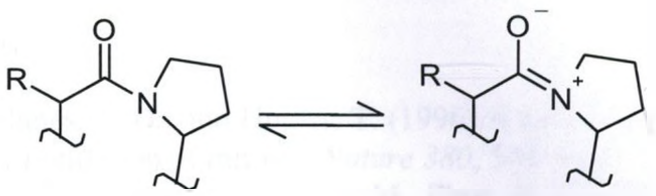
non-polar, single bond form of the N-C bond by strategic charge organization in the PPIase active site. More specifically, we propose that the double character of the peptide bond is reduced by presentation of a full or partial negative charge by C113 to the carbonyl oxygen. Hydrogen bonding to the C113 by neighbouring residues such as H59 or S115, would keep the sulfhydryl group of the cysteine in the negative state. In this way, the active site of Pin1 would distort the peptide bond, decreasing the barrier to catalysis without the formation of a covalent intermediate.

The mechanism presented above predicts that the C113 residue would have a low pKa value in the environment of the active site. Although experimental measurement of the pKa of this residue was hampered by the insolubility of the Pin1 protein at low pH, predicted pKa values can be calculated with PROPKA, a computer program that estimates the ionization state of residues based on their position in known structures (*47*). Using this program with two independent crystal structures of Pin1 (PDBs: 1PIN and 1F8A) we calculated very low pKa's for C113, especially when compared with the only other cysteine in Pin1, C57 (Figure 2.6C). Interestingly, substitution of H59 with L in the 1PIN structure causes a rise in the pKa value, suggesting this residue may contribute to the negative state of C113 in the active site. These predictions demonstrate that the non-covalent catalytic mechanism presented here where bond rotation is aided by a negative charge at C113, is a good hypothetical model that can be used in future testing.

In summary, this study, which examined the function and activity of specific mutations in Pin1, has refined the role for a number of critical residues in both the WW and PPIase domains of Pin1 and has yielded new insights into how the PPIase domain catalyzes the isomerization of the peptidyl-prolyl bond.

Figure 2.6- Proposed non-covalent mechanism for Pin1 catalysis. A) The double bond character of the peptide bond is due to resonance between a more non-polar, tetrahedral form (*left*) and a charged, double bond form (*right*). Chemical environments which enhance the form on the left will have a lower energy barrier for bond rotation. B) In the Pin1 active site, hydrogen bonding to C113 (possible bonds are shown in green) would allow the side-chain sulfhydryl group to present a partial or full negative charge to the carbonyl oxygen of the prolyl bond (bond to be rotated is shown in magenta). This would stabilize the single bond form of the peptide bond enabling bond rotation around the green arrow. Diagram created using ISIS. C) Chart of pKa values predicted using the PROPKA computer program.

A)



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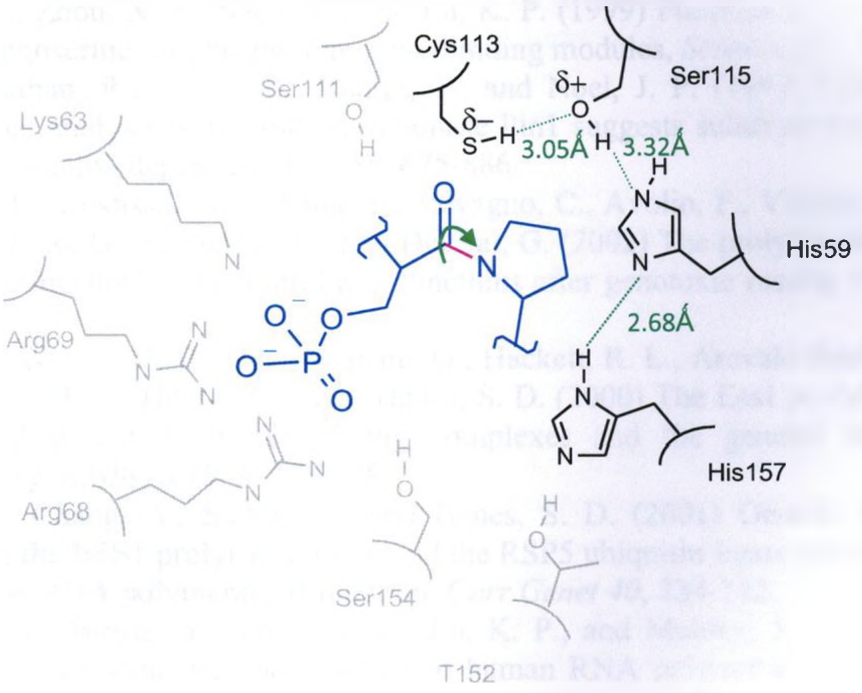

C)

Crystal Structure	pKa's			
	C57	H59	C113	H157
1F8A	8.41	8.51	-1.08	1.21
1PIN	9.05	7.69	-0.64	0.72
1PIN (H59L)	9.03	-	4.99	2.25

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Chapter 3: The dual histidine motif in the active site of Pin1 has a structural rather than catalytic role[1]

3.1 Introduction

Peptidyl-prolyl isomerases (PPIases) are a class of enzymes that catalyze rotation about the peptide bond preceding a proline (*1, 2*). Pin1, a member of the parvulin family of PPIases, is unique among this class of enzymes because of its ability to bind and isomerize phosphorylated sequences suggesting a role for Pin1 in cell signalling (*3-7*). Indeed, Pin1 is involved in many cellular processes including cell growth and division (*4, 8-12*), transcription (*13-16*), DNA damage repair and apoptosis (*17-22*). As such, Pin1 has been shown to interact with a number of phosphoproteins involved in these processes (*5, 10*). Furthermore, the Pin1 homolog, Ess1, is essential in *Saccharomyces cerevisiae (23)* and *Candida albicans (24)* and Pin1 itself is implicated in Alzheimer's disease (*22, 25*) and cancer (*26-29*).

Structurally, Pin1 consists of two domains: an N-terminal WW domain which binds phosphoserine/threonine-proline (pS/T-P) motifs with high affinity and a C-terminal PPIase domain responsible for Pin1 isomerase activity that is shared among all the members of the parvulin family (*6, 7, 30*). Although structures of Pin1 and related parvulins are known, its catalytic mechanism remains elusive. Mutational analysis and inhibitor studies involving the active site residue, C113, have shown that this residue plays a key role in catalysis (*31, 32*). As substitution of C113 with aspartic acid results in

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partially active Pin1, catalysis likely proceeds through a non-covalent mechanism (*31*). However, the roles of other active site residues in Pin1 activity and function are not well understood. Ultimately, a more comprehensive study of residues in the active site of Pin1 is required to better decipher its regulatory role in cells and develop its potential as a therapeutic target.

Previously, our lab used a plasmid shuffling assay in *S. cerevisiae* to screen for residues of human Pin1 that were important for function *(31)*. Surprisingly, a number of conserved residues, including two active site histidines, H59 and H157, could be mutated without impeding function (*31*). This was unexpected as these two histidine residues are absolutely conserved in parvulin PPIase domains. In this study, we use a mutational approach to define the sequence requirements at positions H59 and H157. Our results reveal that these residues do not seem to be important for hydrogen bonding or direct interactions with the substrate, but rather play a key role in the stability of the active site.

3.2 Material and Methods

Construction of Plasmids

The LEU2-containing centromeric pY204 constructs for expressing wild-type, H59L and H157L Pin1 alleles in yeast have been described previously (See Chapter 2, (*31*)). The double H59L/H157L mutant was made by combining NotI/PstI and PstI/EcoRI fragments from H59L and H157L alleles into a digested NotI/EcoRI pY204 vector. Oligonucleotides for other histidine mutations are shown in Table I. Mutations to H59 were made in the pY204-WTPin construct using the Quikchange kit (Stratagene) according to manufacturer's instructions. Mutations to H157 were made through PCR using the Pin1 forward (PinF) primer: 5'-ATA AGA ATG CGG CCG CCA TGG CGG ACG AGG AGA-3' and the appropriate reverse primer with either wild-type Pin1 or the H59L construct as template. PCR products were then ligated into PCR blunt (Invitrogen) and subcloned into the pY204 construct using NotI and EcoRI. Constructs for expression of GST fusion proteins were made by subcloning the mutants using NcoI and HindIII into a pGEX construct with an added TEV cleavage site 5' to the multiple cloning site.

Yeast plasmid shuffling assay and yeast protein expression

The yeast plasmid shuffling assay to determine functionality of the Pin1 alleles was performed after transformation of Pin1-containing alleles into yeast strain YKH100 (genotype, MAT*a* *ura3-1 leu2-3, 112 trp1-1 can1-100 ade2-1 his3-11*, 15[phi+] *ess1Δ::TRP1*; (*31*))as described previously (*31*). To evaluate expression of Pin1 derivatives, yeast were lysed by bead lysis (*31*). Crude protein lysates were separated by SDS-PAGE and Western blotted with anti-FLAG M2 (Sigma).

Table 3.1- Oligonucleotides used in site-directed mutagenesis in Chapter 3

Name	Sequence
H59A Forward	5'-GTC CGC TGC TCG GCC CTG CTG GTG AAG-3'
H59A Reverse	5'-CTT CAC CAG CAG GGC CGA GCA GCG GAC-3'
H59N Forward	5'-GTC CGC TGC TCG AAC CTG CTG GTG AAG-3'
H59N Reverse	5'-CTT CAC CAG CAG GTT CGA GCA GCG GAC-3'
H59F Forward	5'-GTC CGC TGC TCG TTC CTG CTG GTG AAG-3'
H59F Reverse	5'-CTT CAC CAG CAG GAA CGA GCA GCG GAC-3'
H59S Forward	5'-GTC CGC TGC TCG AGC CTG CTG GTG AAG-3'
H59S Reverse	5'-CTT CAC CAG CAG GCT CGA GCA GCG GAC-3'
H157A Reverse	5'-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GGC GAT GCC GGA-3'
H157N Reverse	5'-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GTT GAT GCC GGA-3'
H157F Reverse	5'-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GAA GAT GCC GGA-3'
H157S Reverse	5'-GGA ATT CTC TCA CTC AGT CGC GAG GAT GAT GCT GAT GCC GGA-3'

GST fusion protein purification

GST fusion proteins were expressed from pGEX constructs transformed into *E. coli* strain BL21. Individual colonies were grown in L Broth with 100 μg/mL ampicillin (Roche) at 37 °C then induced overnight at 18°C with 0.6 mM isopropylthio-α-D-β-galactoside. Bacteria were pelleted and resuspended in PBS containing protease inhibitors (1mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A and 10 μg/mL leupeptin) and then lysed by sonication before mixing with 1% Triton X-100 for 15 min at 4 °C. Bacterial lysates were cleared by centrifugation and GST fusion proteins were bound to glutathione cross-linked agarose beads (Sigma) and eluted in 10 mM reduced

glutathione (Sigma) in PBS. Proteins were analysed by SDS-PAGE to assess purity and then dialysed into PBS containing 20% glycerol for storage.

Cleavage of GST fusion proteins

Proteins used for trypsin and CD experiments were expressed as GST fusions and purified as above, but without dialysis into storage buffer. Instead, proteins were cleaved with TEV protease for four hours at room temperature and dialysed overnight into Buffer A (10 mM NaH_2PO_4, 15% glycerol, 5 mM NaN_3, 20 mM β–mercaptoethanol, pH 6.5). Proteins were loaded onto a cation exchange column (HiTrap SP HP, GE Healthcare), washed and eluted over a gradient of Buffer B (10 mM NaH_2PO_4, 15% glycerol, pH 11). Alternatively, for experiments performed in Tris buffer, proteins were loaded onto the cation exchange column and washed with Buffer A, then exchanged into 10 mM Tris-HCl, 10 mM sodium acetate, 15% glycerol, pH 7.5 and eluted over a gradient of 10 mM Tris-HCl, 10 mM sodium acetate, 15% glycerol, pH 9. Proteins were analysed for purity by SDS-polyacrylamide gel electrophoresis. Pin1-containing fractions were pooled, buffer exchanged to pH 7.5 where necessary and concentrated for analysis.

In vitro isomerase assays

Isomerase activity was measured as described previously (*31*). Assays were performed at 0 °C in a Cary-100 spectrophotometer. Briefly, the AEPF-*p*NA or Pintide (WFYpSPR-*p*NA) substrate (Bachem) was added to assay buffer (50 mM Hepes, 100 mM NaCl, 5 mM NaN_3, pH 7.5) before the addition of chymotrypsin or trypsin (Sigma). Cleavage of the *p*NA group from the substrate was followed at either 390 nm, 405 nm, 430 nm or 445 nm depending on substrate concentration. Once the *trans* form of the substrate was cleaved, the chemical rate of catalysis was recorded before addition of wild-

type or mutant Pin1. Rates of reaction for 4 to 7 different substrate concentrations were recorded, corrected for the rate of chemical isomerisation and enzyme concentration, and plotted against substrate concentration to determine the k_{cat}/K_M.

Partial trypsin proteolysis

For trypsin digestion, 100 μg of recombinant protein was digested with 0.5μg of trypsin (Sigma) in 200 μL of 10 mM NaH_2PO_4, 15% glycerol, pH 7.5 at room temperature. Aliquots of 20 μL were taken at indicated times and the reaction stopped by addition of SDS sample buffer and boiling. Samples were analysed by SDS-PAGE and bands visualised after staining with Coomassie Brilliant Blue R-250 (Bio-Rad).

Circular dichroism measurements

CD measurements were performed in 10 mM NaH_2PO_4, 15% glycerol pH~7.5 on a Jasco J-810 spectropolarimeter. Scans were taken using a 0.1 mm path length cuvette with protein concentrations between 0.736 mg/mL and 0.934 mg/mL over a temperature range of 20 °C-80 °C.

GST pull-downs

Mitotically-arrested U2OS osteosarcoma lysates were made by treating cells with 0.06 μg/mL of nocodazole (Sigma) for 18 hours. Cells were harvested by collecting cells loosely attached to the tissue culture plate and resuspending them in NP-40 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40) containing 1 mM PMSF, 10 μg/mL pepstatin A, 10 μg/mL leupeptin and 1 μM okadaic acid, lysed by sonication and cell debris pelleted by centrifugation. For GST pull-downs, 100 μg of GST fusion protein was bound to glutathione cross-linked to agarose beads (Sigma). Beads were washed with PBS buffer then incubated with 1mg of U2OS lysate for 1 hour at 4 °C. Beads were

washed with 400 μL NP-40 buffer and bound protein eluted into SDS sample buffer by boiling. Proteins were analysed by SDS-PAGE and Western blotted with MPM-2 antibody (Upstate Biochemistry).

3.3 Results

Mutation of H59 and H157 in Pin1 support viability in yeast

Pin1 contains two histidine residues, H59 and H157 that had been postulated to be important for Pin1 function as they are absolutely conserved in parvulin PPIase domains (Figure 3.1A). Furthermore, these two residues lie side-by-side in the active site and H59 specifically is within hydrogen-bonding distance of the catalytic C113 residue of Pin1. Previously, we performed a unigenic evolution analysis of human Pin1 by selecting for mutations that would support viability in the yeast *S. cerevisiae*. As part of this analysis, we found that H59 could be altered to Q without affecting viability; however, alteration to L led to inviability. In contrast, altering H157 to L supported viability. Accordingly, we hypothesized that H59 and H157 are involved in catalysis by stabilizing the transition state through hydrogen bonding to the substrate carbonyl oxygen (*31*).

As a logical extension of our earlier work, we tested this hypothesis by performing a more detailed investigation of these two active site histidines. Single mutations to H59 and H157 were generated and their function in maintaining yeast viability (as determined by their growth on plates containing 5FOA) was evaluated. Although these two histidines are well conserved and it was expected that their mutation would be detrimental to Pin1 function, many mutations were tolerated in yeast at these two positions (Figure 3.1B). For residue 59, mutations to large hydrophobic residues, such as leucine and phenylalanine, displayed significantly reduced growth in yeast compared to wild-type Pin1. However, substitution of H59 with the smaller hydrophobic residue, alanine, supported viability. Unlike H59 mutations where only some substitutions were tolerated, all of the single mutations to H157 supported viability (Figure 3.1B), suggesting that

H157 is not as important in Pin1 function in yeast as H59. Together our results indicate that while highly conserved, neither of the histidines are essential for viability.

Double mutation of active site histidines restores viability in yeast

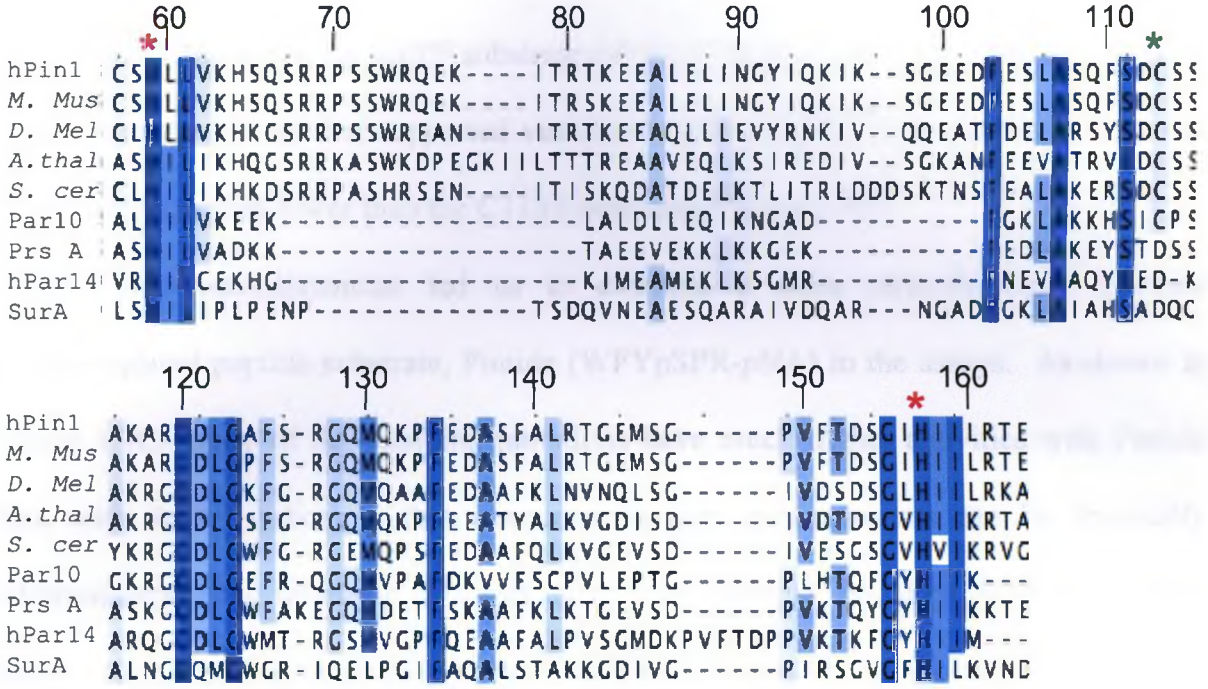
During the analysis of single mutations, we serendipitously engineered a double mutation containing H59L and H157L. Surprisingly, in contrast to the single H59L mutation, the double mutation supported viability (Figure 3.2A) suggesting that the H157L mutation rescues Pin1 activity. To examine further how this rescue was occurring, various double mutations where H157 was mutated in the presence of H59L were examined for their ability to support viability in yeast. These studies revealed that other hydrophobic substitutions to H157, including phenylalanine and alanine, can rescue Pin1 function in the presence of H59L (Figure 3.2C). By comparison, the polar H157S mutation could only partially rescue the H59L mutation while the mutation of H157N was unable to rescue. Collectively, these results indicate that a combination of hydrophobic residues at positions 59 and 157 will support viability.

H59 and H157 mutants have isomerase activity against a phosphorylated substrate in vitro

It was unexpected that mutations to H59 and H157 would be so well tolerated in yeast because of their placement within the active site and their conservation in parvulin PPIase domains. To further examine how these residues are involved in Pin1 and their role in the active site, recombinant proteins for each of the mutations were purified (Figure 3.3A) and their activity compared using an *in vitro* isomerase assay. To enable rapid purification, proteins were expressed as GST fusions. Tests with the wild-type Pin1 GST fusion and a cleaved version of the protein without the tag show that the presence of GST is not detrimental to isomerase activity (Figure 3.3B). Initial results with an

Figure 3.1- Mutations to conserved residues H59 and H157. A) Sequence alignment of Pin1 and parvulin PPIase domains from various organisms. The conserved Histidine residues H59 and H157 (in human Pin1) are conserved in all species and indicated by red asterisks. C113 is indicated by a green asterisk. Residues are numbered according to human Pin1. Alignment was done using Clustal W. B) Site-directed mutation of conserved residues H59 and H157. Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density and serial dilutions were spotted onto 5FOA plates to monitor growth. Mutations were classified as either functional with growth comparable to wild-type Pin1 (+) or non-functional with significantly reduced growth compared to wild-type Pin1 (-) in rescuing the ess1::TRP1 disruption after being spotted onto 5FOA

A)



B)

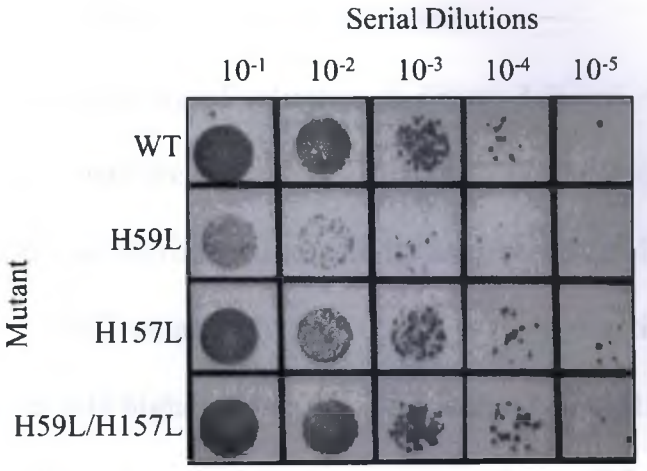
X=	H59X	H157X
L	-	+
A	+	+
N	+	+
F	-	+
S	+	+

unphosphorylated AEPF peptide substrate found the non-functional C113S mutation had 3.4% of wild-type activity and the functional histidine mutations had activities which were undetectable for H59L/H157L, but were as high as 38.5% for H157S (Figure 3.3C). Clearly, activities using the AEPF substrate did not correlate well with our results in yeast as many of the alleles that supported viability (H59A, H59L/H157L, H59L/H157F and H59S) had activities lower than the C113S mutation (Figure 3.3C).

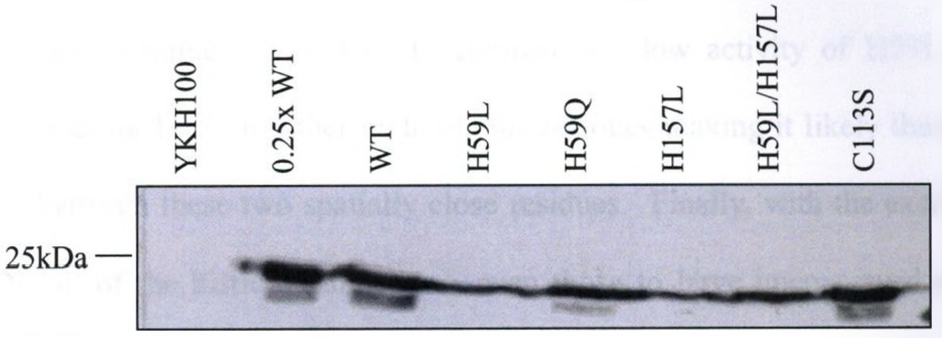
These inconsistencies led us to examine a more physiologically relevant phosphorylated peptide substrate, Pintide (WFYpSPR-*p*NA) in the assays. As shown in Figure 3.3C, many of the histidine mutations have much higher activities with Pintide than with AEPF indicating that isomerase activity measurements can be drastically different depending upon the substrate used. For example, the H59L/H157A mutation has less than 0.1% of wild-type activity with AEPF, but 41.2% of wild-type activity with Pintide. As well, results from assays using Pintide show that the low activity of the H59L mutation can be increased almost 5-fold when combined with a mutation of H157L (Figure 3.3C). This is consistent with results in yeast where mutations of H157L could rescue the inviable H59L mutation (Figure 3.2A). With Pintide as substrate, isomerase activity of the remaining mutations also correlated well with yeast plasmid shuffling results (including C113S which had an activity of only 0.81% with Pintide). The ordering of mutations by their activity in Figure 3.3 indicates that at least 5% of wild-type Pin1 activity is needed to maintain viability in yeast in this system. However, the exact amount of activity needed is difficult to determine precisely as expression levels of the mutants in yeast can vary slightly (Figure 3.2B). For example, H59L/H157L had a higher level of expression in yeast than H59L, but it also had 5-fold more activity suggesting that

Figure 3.2- Mutation of H157 rescues the inviable H59L mutation in *S. cerevisiae.* A) Individual mutants were transformed into yeast and plasmid shuffling was performed. Transformants were normalized according to cell density and serial dilutions were spotted onto 5FOA plates to monitor growth. Those mutants which grew robustly on 5FOA were considered to be functional in yeast cells. B) Expression of Pin1 plasmids in yeast. Cultures of each mutant were grown in SC –W-L-U media overnight and lysed using glass beads. One hundred twenty-five micrograms of each lysate were run on 12% SDS-PAGE transferred and blotted with anti-FLAG antibody. In the 0.25x WT lane, only 31.25 μg of lysate was loaded. C) Site-directed mutations were made to H157 in the presence of H59L and tested in the yeast plasmid shuffling assay as in A). Mutations were classified as either functional (+), non-functional (-) or partially functional (+/-) in rescuing the ess1::TRP1 disruption after being spotted onto 5FOA.

A)



B)



C)

X=	H59L/157X
L	+
A	+
N	-
F	+
S	+/-

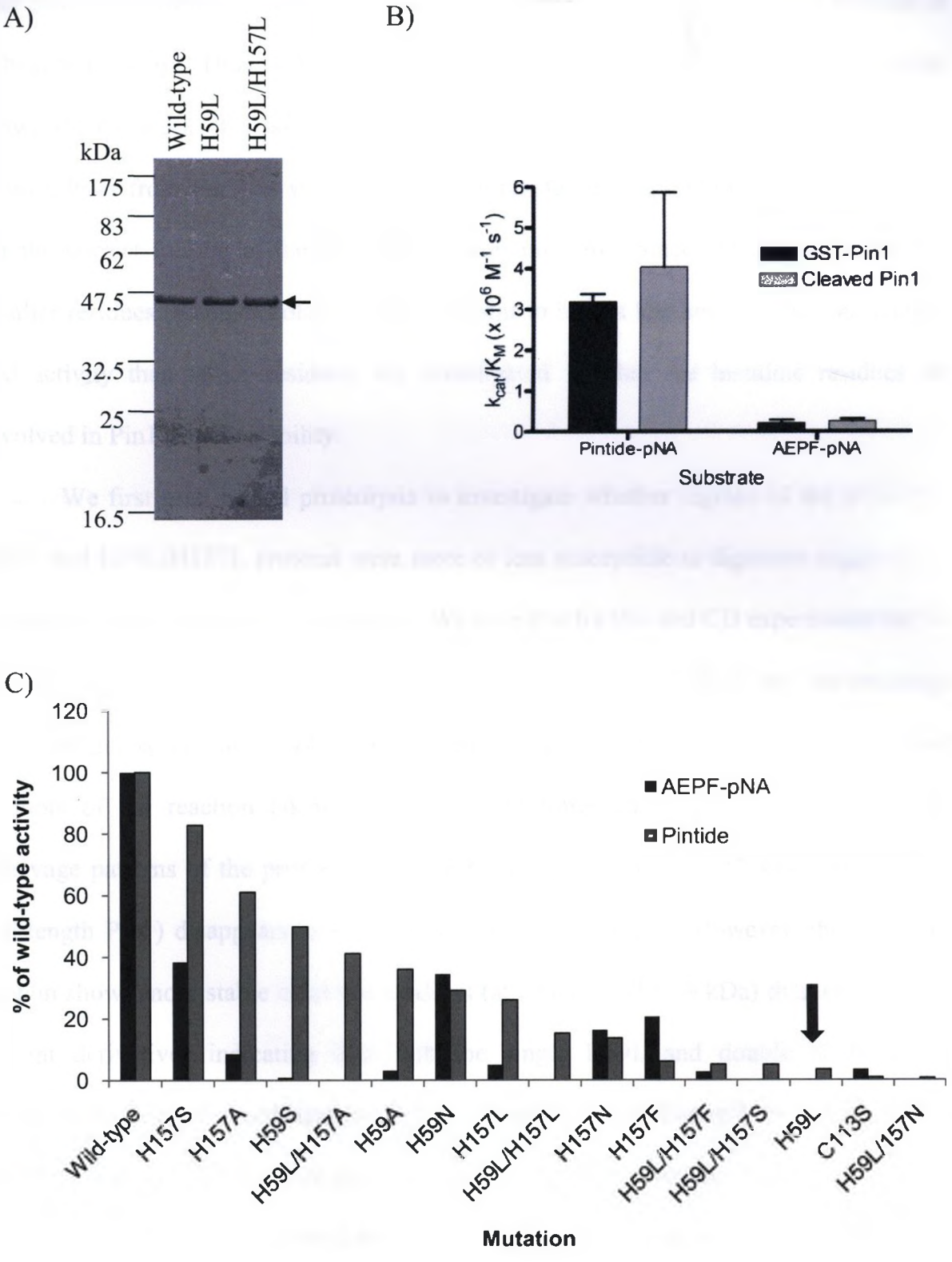
both of these factors may contribute to the rescue of the inviable H59L mutation by the second H157L mutation seen in our yeast assay.

The rank ordering of activities in Figure 3.3C also reveals a number of general trends about the histidine mutations. First, as with the yeast results, mutations of H157 are not generally as detrimental to activity as mutation of H59 to the same residue. For example, the H59A mutation had 36.0% of wild-type activity whereas H157A activity was almost two-fold higher at 61.0%. This suggests that H157 is less critical for enzyme function than H59. A second observation from Figure 3.3 is that mutation of either H59 or H157 to small residues (A, N, or S) is less disruptive to Pin1 activity than mutation to larger hydrophobic residues (L or F). In contrast, the low activity of H59L can be increased by mutating H157 to other hydrophobic residues making it likely that there is an interaction between these two spatially close residues. Finally, with the exception of H59L/H157N, all of the histidine mutations, even those to large unconserved residues, had higher activity than C113S. This suggests that although these two histidines are in the active site, they are not as important as C113 for catalysis, at least with a phosphorylated substrate. Since Schutkowski et al. observed dramatic changes in activity by lowering pH suggesting that ionization of the active site histidines influenced catalysis (*33*), we also examined the activities of H157A and H157N at lower pH (pH 6). In these assays, the behaviour of the mutants in response to the changes in pH did not differ from the wild-type enzyme (data not shown).

Mutation of active site histidines affects Pin1 stability

The double H59L/H157L mutation clearly has more activity than a single H59L. As the rescue of the H59L mutation by alteration of H157 to L is inconsistent with our proposed catalytic mechanism that requires side-chain hydrogen bonding at these

Figure 3.3- *In vitro* isomerase activities of Pin1 recombinant proteins. A) Coomassie-stained SDS-PAGE of proteins after purification of GST fusion proteins. B) Comparison of *in vitro* isomerase activities of purified wild-type Pin1 proteins before and after cleavage of the GST tag using APEF-*p*NA and Pintide-*p*NA substrates. C) Comparison of the *in vitro* isomerase activities of histidine mutants using AEPF-*p*NA and Pintide-*p*NA peptide substrates. Histidine mutations are ordered according to their isomerase activities using Pintide. The division between the functional and non-functional mutations is marked with an arrow.


A)
Wild-type
H59L
H59L/H157L
kDa
175
83
62
47.5
32.5
25
16.5
B)
k_{cat}/K_M (x 10^6 M^{-1} s^{-1})
GST-Pin1
Cleaved Pin1
Pintide-pNA
AEPF-pNA
Substrate
C)
% of wild-type activity
AEPF-pNA
Pintide
Wild-type
H157S
H157A
H59S
H59L/H157A
H59A
H59N
H157L
H59L/H157L
H157N
H157F
H59L/H157F
H59L/H157S
H59L
C113S
H59L/157N
Mutation

positions (*31*), these residues must have another non-catalytic function. NMR data shows that these two histidines have only slight changes in chemical shift upon the addition of substrate (*34-36*). Thus, H59 and H157 are likely not involved in substrate binding. However, the work of Tossavainen *et al.* (2006) with another member of the parvulin family, PrsA from *Bacillus subtilis*, shows the residue corresponding to H59 is essential for the correct folding of the PrsA PPIase domain (*36*). Since our data indicates that smaller residues (whether polar or apolar) appear to have a less severe effect on function and activity than larger residues, we investigated whether the histidine residues are involved in Pin1 protein stability.

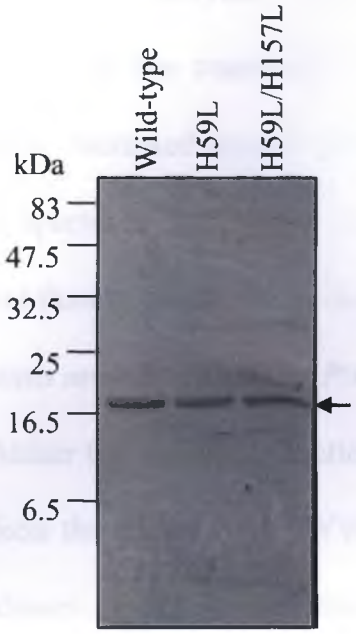
We first used partial proteolysis to investigate whether regions of the wild-type, H59L and H59L/H157L proteins were more or less susceptible to digestion suggesting a change in protein structure or dynamics. We note that for this and CD experiments the N-terminal GST was removed and the proteins further purified by ion-exchange chromatography (Figure 3.4A). Pin1 derivatives were incubated with trypsin, and aliquots of the reaction taken at the indicated times and analyzed by SDS-PAGE. Cleavage patterns of the proteins reveal that the highest band at ~17 kDa (representing full-length Pin1) disappears at a similar rate for each protein. However, the wild-type protein shows more stable cleavage products (at ~11 kDa and ~9 kDa) than either of the mutant derivatives indicating that both the single H59L and double H59L/H157L mutations lead to enhanced susceptibility to trypsin cleavage (Figure 3.4B). Interestingly, the ~9 kDa band is more stable for the H59L/H157L derivative from 20-50 minutes than for the H59L derivative (Figure 3.4B) although why this band should be more stable with an H157L mutation could not be determined from our partial proteolysis data. This suggests that the low activity of H59L may be due in part to a change in the protein that

renders it more susceptible to trypsin cleavage and that this defect can be partially rescued by a mutation of the second histidine to leucine.

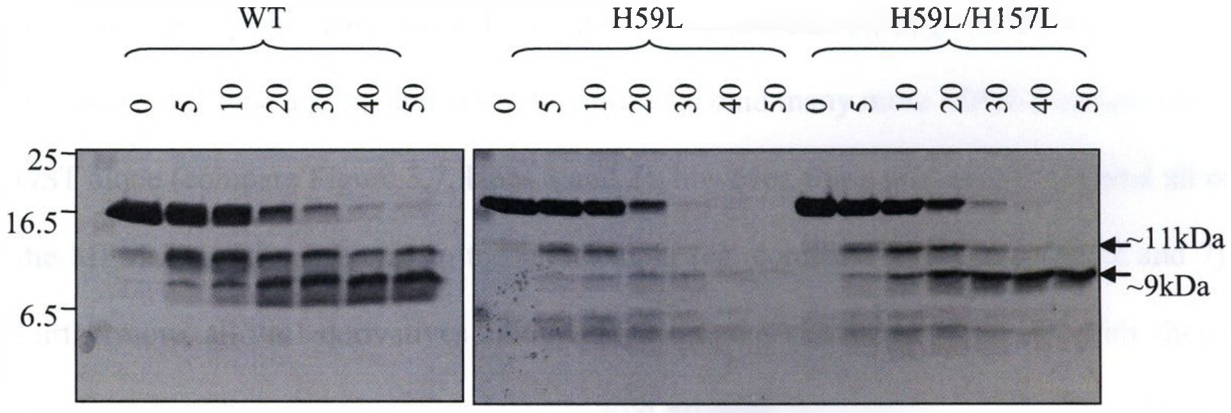
To further test the stability of Pin1 proteins, we used Far-UV CD to examine the global structures of the proteins at different temperatures. CD spectra of the H59L and H59L/H157L proteins both overlay on the wild-type Pin1 spectrum at 20 °C indicating that these proteins have similar global folds at this temperature (Figure 3.5A). Spectra collected at higher temperatures reveal that while wild-type Pin1 has a stable spectrum up to at least 50 °C, both proteins containing histidine mutations have altered spectra and are destabilized at temperatures above 30 °C (Figure 3.5B). To further examine the thermal denaturation of each of these proteins, their CD signal at 200 nm was monitored over a temperature range from 20 °C-70 °C. The 200 nm wavelength was chosen because the change in CD signal of the WW domain was minimal over this temperature range while the PPIase domain where we made our mutations showed a significant shift (Supplementary Figure 3.1). Denaturation of Pin1 proteins was not reversible (data not shown), so CD spectra were used as a comparative assessment between different proteins. These curves revealed that both H59L and H59L/H157L start to decrease in signal at much lower temperatures than wild-type Pin1 (Figure 3.6), but the H59L mutation appears to lose signal before H59L/H157L. To evaluate if buffer composition differentially affected the CD profiles of the mutants, thermal denaturation was performed for proteins purified in buffer lacking phosphate or sulphate. While the Pin1 proteins were less stable in the Tris-acetate buffer, the rank order of different mutations were the same in both buffers (Figure 3.6). Although the data collected do not allow for an exact interpretation of how histidine mutations affect Pin1 structure, it does suggest that Pin1 containing H59L is unstable at higher temperatures and that alteration of H157L can help

Figure 3.4- Partial proteolysis of Pin1 proteins. A) Coomassie-stained SDS-PAGE of proteins after cleavage from the GST tag. B) Partial trypsin proteolysis. Trypsin was added to wild-type or leucine mutant proteins in a 1:200 ratio and incubated at room temperature. Aliquots were taken at stated times analysed by SDS-PAGE and stained with Coomassie Blue.

A)


Wild-type
H59L
H59L/H157L
kDa
83
47.5
32.5
25
16.5
6.5

B)


WT
H59L
H59L/H157L
0
5
10
20
30
40
50
25
16.5
6.5
~11kDa
~9kDa

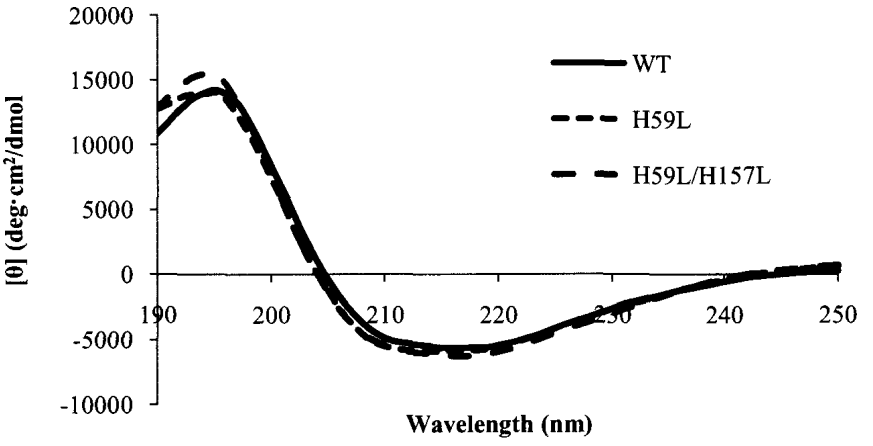
stabilize the H59L form of the enzyme. As with the trypsin proteolysis, these results point to a structural role for the conserved histidines. However, the result that the histidine mutations cause increased trypsin proteolysis at room temperature, but do not display changes in CD spectra at 20 °C suggests that these residues may affect the protein dynamics of Pin1 rather than its overall structure (see Discussion).

PPIase domain mutations are not critical for Pin1 target interactions

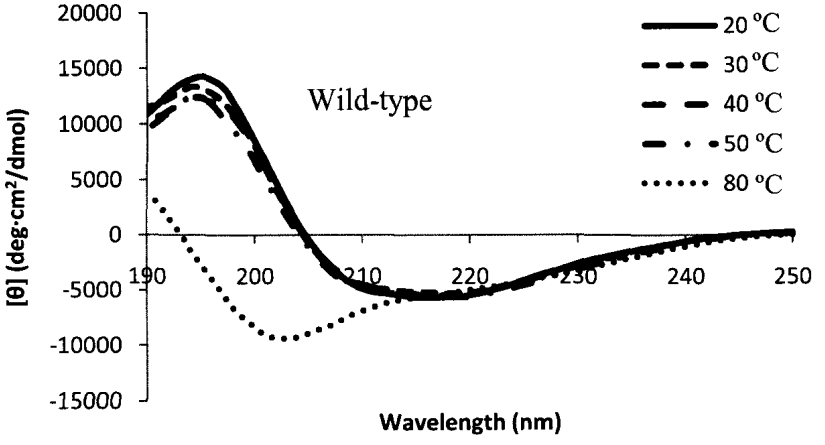
To address whether the impaired function of the PPIase domain due to mutations in H59 and H157 affects the ability of the WW domain to bind interacting partners, we performed GST pull-downs. Extracts from mitotically-arrested U2OS cells were applied to glutathione beads on which were immobilized GST fusion proteins of Pin1 derivatives. Beads were washed and bound proteins analyzed by Western blotting with anti-MPM-2, an antibody raised against a mitotic HeLa lysate which has been shown to recognize many phosphorylated epitopes and Pin1-interacting proteins (*4, 37*). As shown in Figure 3.7, both wild-type and mutant GST-Pin1 proteins bind many more MPM-2 antigens than GST alone (compare Figure 3.7, lanes 1 and 2); however, these proteins do not bind all of the MPM-2 reactive proteins in the initial input (eg. compare Figure 3.7, lane 2 and 7). Furthermore, all Pin1 derivatives bind MPM-2 antigens similar to wild-type. This shows that Pin1 histidine mutations can interact with proteins in a manner similar to wild-type Pin1. As most of these interactions occur through the WW domain of Pin1, Figure 3.7 demonstrates that the integrity of the WW domain is not affected by the PPIase mutations nor does a non-functional mutation in the PPIase domain (eg. H59L) appear to affect the ability of the WW domain to bind interacting partners.

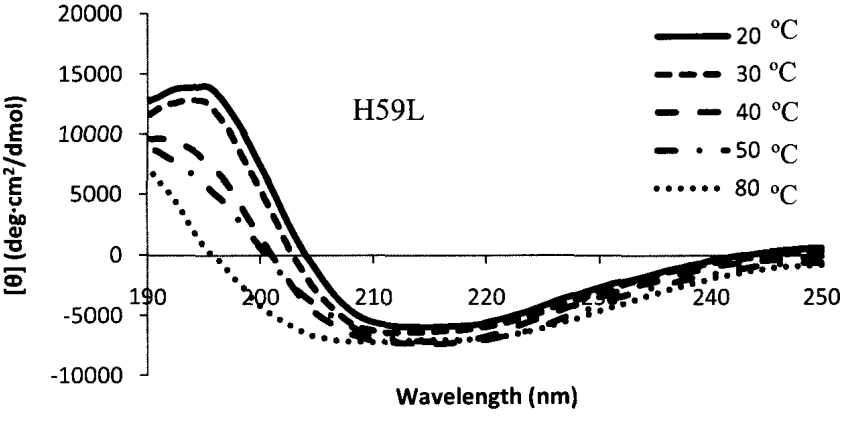
Figure 3.5- Far-UV CD of Pin1 proteins. A) Far-UV spectra of Wild-type, H59L and H59L/H157L proteins at 20 °C. B) CD spectra of Wild-type, H59L and H59L/H157L proteins at stated temperatures.

A)


20000
15000
10000
5000
0
-5000
-10000
190
200
210
220
230
240
250
[θ] (deg·cm²/dmol)
Wavelength (nm)
WT
H59L
H59L/H157L

B)


20000
15000
10000
5000
0
-5000
-10000
-15000
190
200
210
220
230
240
250
[θ] (deg·cm²/dmol)
Wavelength (nm)
Wild-type
20 °C
30 °C
40 °C
50 °C
80 °C


20000
15000
10000
5000
0
-5000
-10000
190
200
210
220
240
250
[θ] (deg·cm²/dmol)
Wavelength (nm)
H59L
20 °C
30 °C
40 °C
50 °C
80 °C

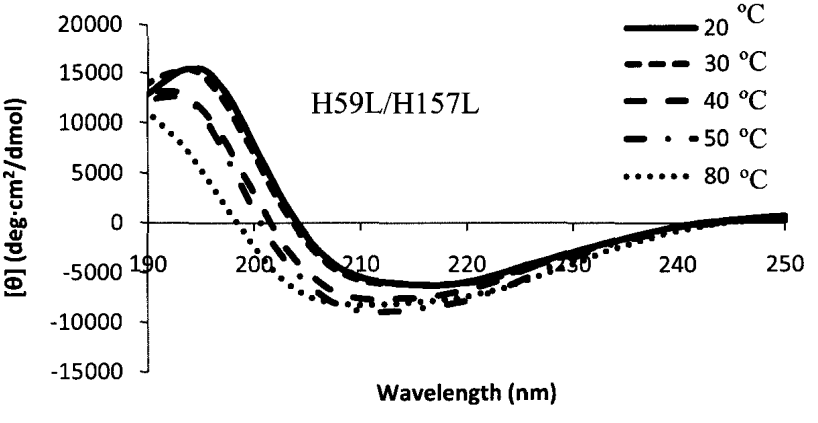
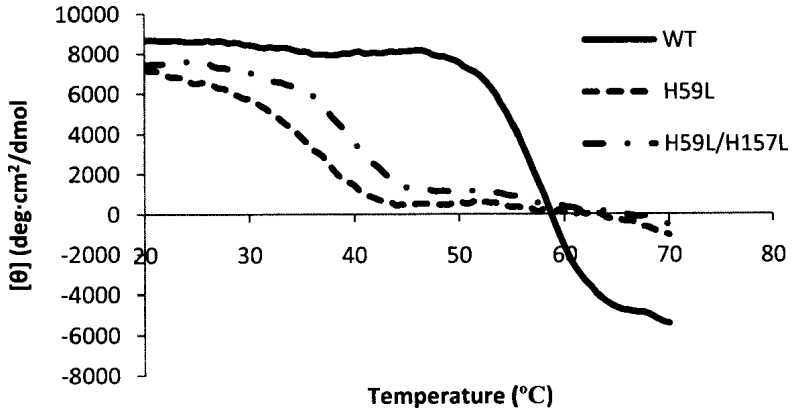
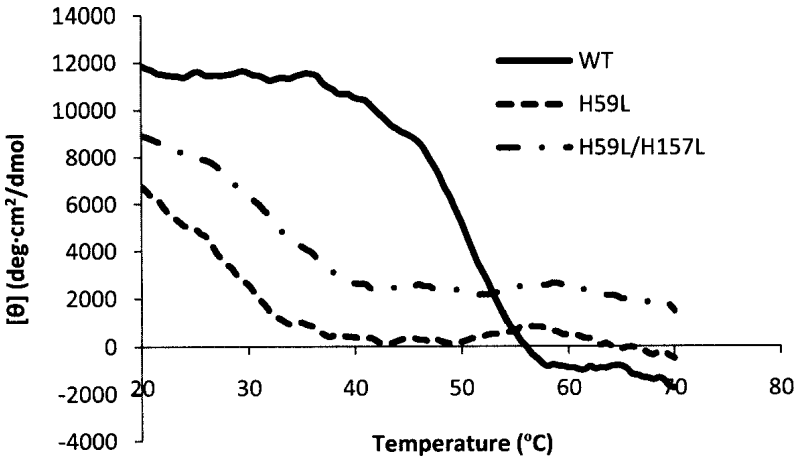

20000
15000
10000
5000
0
-5000
-10000
-15000
190
200
210
220
240
250
[θ] (deg·cm²/dmol)
Wavelength (nm)
H59L/H157L
20 °C
30 °C
40 °C
50 °C
80 °C

Figure 3.6- Thermal denaturation curves of Pin1 wild-type and mutant proteins. Readins were taken at 200 nm in A) phosphate and B) Tris buffers.

A) Phosphate buffer



B) Tris buffer

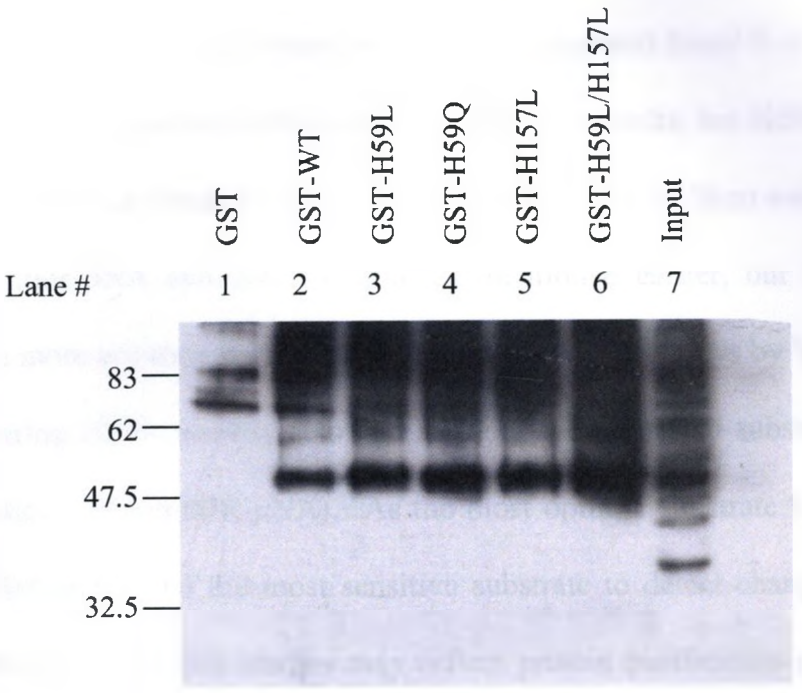


3.4 Discussion

Many of the amino acid substitutions to H59 and H157 supported viability in yeast. The finding that these functional mutations had a range of activities *in vitro* is not unexpected as in *S. cerevisiae*, only a small amount of isomerase activity is needed to support viability (*38*). Isomerase activities with the Pintide (WFYpSPR-*p*NA) substrate correlated well with the ability of mutants to support viability demonstrating that phosphorylation is important for Pin1 function *in vivo*. Approximately 5% of wild-type activity was sufficient to maintain viability in yeast. Results show that assays containing AEPF substrate had different activities that did not correlate well with results in yeast (Figure 3.3). This may be because the more unstable mutant proteins could not bind an E-P motif as well as the preferred pS-T motif. Alternatively, the Pintide substrate is longer than AEPF by an extra two amino acids which may have allowed it to make more contacts outside of the active site that helped in its binding to the PPIase domain. Another possibility is that either the active site histidines or the phospho-motif of the substrate may be involved in acid/base reactions which are required for isomerization. Overall, as assays using Pintide show a much better correlation to yeast function than those of AEPF, Pintide is a much more relevant substrate for the interpretation of Pin1 activity.

The C113 residue of Pin1 is thought to be critical for catalysis (*6, 31, 32*). Due to their close proximity, both active site histidines, especially H59, were also thought to play a role in catalysis (*6, 31*). Since most substitutions at H59 and H157 had greater activity than C113S, the histidines cannot be as important for activity. The observation that the alanine set of mutations have significant activity suggests that hydrogen bonding at these

Figure 3.7- Pull-downs with recombinant GST Pin1 proteins. Nocodazole-treated U2OS lysates were incubated with GST-Pin1 wild-type or Leucine mutant proteins bound to glutathione beads. Bounds interactors were blotted with anti-MPM-2.


GST
GST-WT
GST-H59L
GST-H59Q
GST-H157L
GST-H59L/H157L
Input
Lane #
1
2
3
4
5
6
7
83
62
47.5
32.5

positions is not important for isomerization. That double hydrophobic mutations, such as H59L/H157L, had significant isomerase activity and could support viability in yeast eliminates the possibility that there is functional redundancy at these positions.

Other studies in which these residues were mutated found that H157A had 92% of wild-type isomerase activity (*39*), consistent with our results, but H59A had only 5.8% of wild-type activity or lower (*4, 11*). However, the assays by Shen and colleagues did not use the Pintide-*p*NA substrate (*4*) and, as mentioned earlier, our results suggest that Pintide is a more accurate indicator of Pin1 function. The assays by Yaffe and colleagues (*11*) measuring H59A activity also had a slightly less optimal substrate (AApSPR-*p*NA versus Pintide - WFYpSPR-*p*NA). As the most optimal substrate known for isomerase activity, Pintide may be the most sensitive substrate to detect changes in Pin1 activity. Other differences between studies may reflect protein purification procedures or buffer conditions which affect protein instability caused by these mutations.

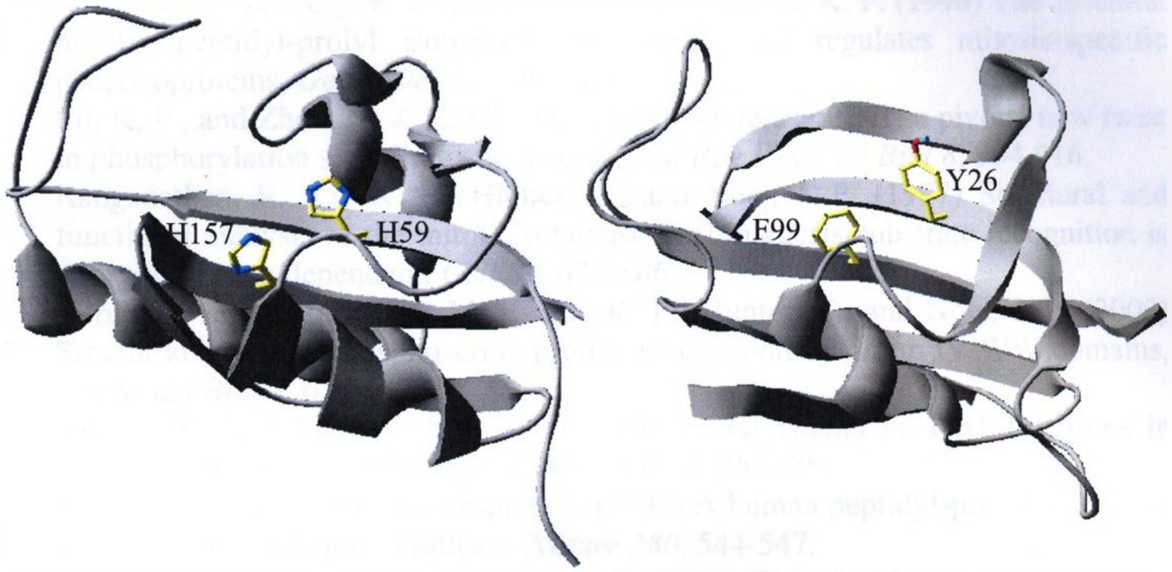
Partial proteolysis and CD analysis of mutant proteins support a model whereby H59 and H157 are important for the integrity of the Pin1 active site. Histidines H59 and H157 are on two adjacent antiparallel β-strands, central in both the four-strand β-sheet and the structure of the PPIase domain (*6, 30*). Due to their central location, it is possible that these two histidines have a key role in the assembly of the sheet/domain structure, supporting the integrity of the active site. As mutations to H59 seem to be more severe than those of H157, it is likely that H59 assumes a more critical and perhaps a dual role, yet the partial rescue of H59L protein instability with an H157L mutation suggests there is structural interaction between these two residues. Exactly how these mutations cause the instability seen in the trypsin digests and denaturation experiments is unclear.

Notably, the CD spectra at 20°C demonstrate that these proteins have the same global structure at this temperature. However the enhanced susceptibility of the mutant proteins to proteolytic cleavage at room temperature does reveal one or more structural differences. This suggests that the effects of the histidine mutations may more accurately be described as changing the protein dynamics of Pin1 rather than the global structure. Several recent studies have examined Pin1 protein dynamics and indicate that certain regions of Pin1 structure may show a degree of flexibility (*40-42*). In this respect, mutations to the central histidines may result in perturbations that cause structural fluctuations that are not reflected in the overall average structure of Pin1. For example, the study by Namanja and colleagues reveals the importance of the flexibility of side chains in the active site of Pin1 in substrate recognition and function (*42*). It is possible that mutations to the dual histidine motif in our work may disrupt the mobility of neighbouring side chains and this could affect both Pin1 stability and catalysis. Alternatively, the phosphospecific-binding loop that contains multiple basic residues (K63, R68 and R69) directly follows the β-strand that contains H59 (*6, 7*). This loop has been crystallized in two conformations, reflecting a certain amount of flexibility. Mutations of H59 to L may increase this flexibility further exposing the basic residues in the loop and making them more sensitive to trypsin.

The PPIase domain of Pin1 is part of the FKBP superfold family (*6*). FKBP12, a representative member of the family, has two residues, a tyrosine (Y26) and a phenylalanine (F99), at the same positions as H59 and H157 (Figure 3.8). Y26 and especially F99 are important in FKBP12 binding and catalysis (*43, 44*). Interestingly, an F99Y mutation has an altered far-UV CD spectrum at 25°C (*43*). Both F99 and Y26 lie

on two β-strands (β2 and β5) that form part of the "folding nucleus" needed to stabilize the transition state structure during folding (*45*), but as the β-sheet of FKBP12 has an extra strand and its active site is much more hydrophobic than the active site of Pin1, the exact interactions involved at these positions may not be the same as those at H59 and H157. It is also interesting to note that upon superposition of the Pin1 and FKBP12 PPIase domains, the catalytic C113 residue of Pin1 which can also be an aspartic acid in some parvulin domains (Figure 3.1A) corresponds to D37 of FKBP12 suggesting that it may have a role similar to C113 in the catalysis of isomerization. By comparison, we propose that H59 and H157 play a role in the folding of the PPIase domain.

Figure 3.8- Comparison of H59 and H157 of Pin1 with the FKBP12 domain. Ribbon diagrams of the Pin1 PPIase domain (left; PDB code: 1PIN) and FKBP12 (right; PDB code: 1FKB) are shown. Residues H59 and H157 of Pin1 lie in the same positions as Y26 and F99 of FKBP12. Y26 and F99 lie in regions of the protein shown to be part of the folding nucleus of FKBP12.


H157
H59
Y26
F99

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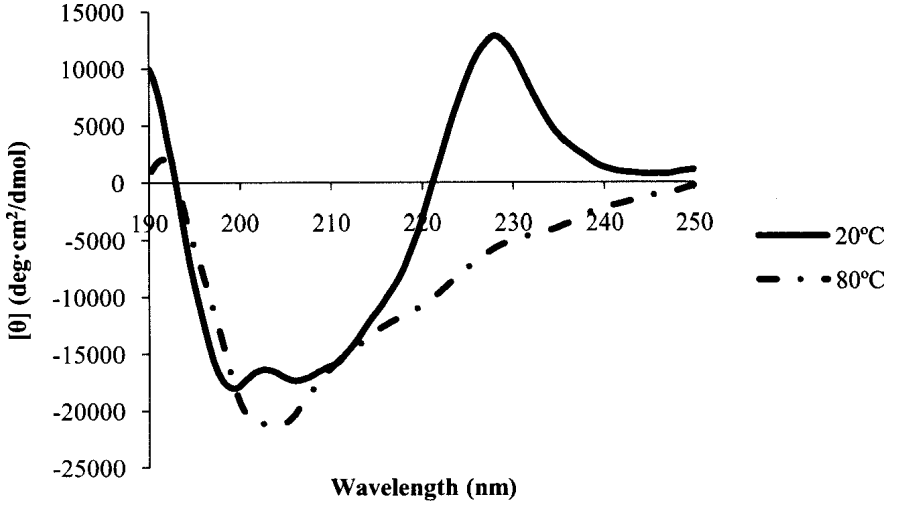
Supplementary Figure 3.1- Selection of the 200nm wavelength for thermal denaturation. CD spectra of the A) WW and B) PPIase domains alone at 20 °C and 80°C. C) Thermal denaturation curves of wild-type and mutant proteins taken at 200nm in phosphate buffer.

A)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[θ] (deg·cm²/dmol)
Wavelength (nm)
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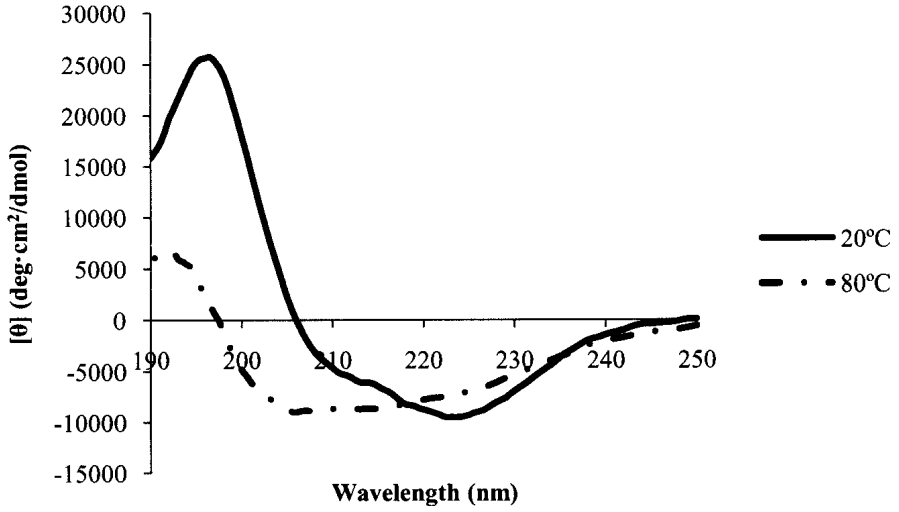
80°C

B)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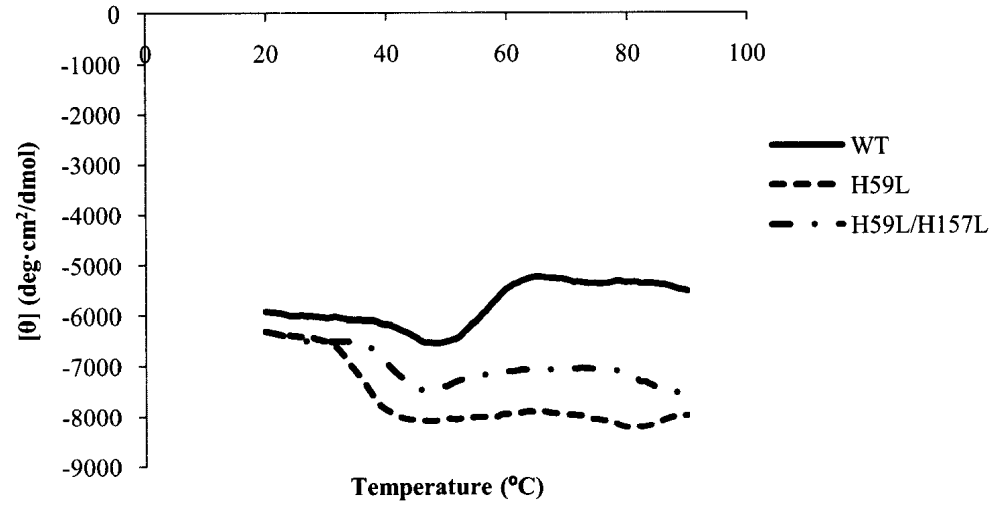
[θ] (deg·cm²/dmol)
Wavelength (nm)
20°C
80°C

C)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-4000
-5000
-6000
-7000
-8000
-9000
0
20
40
60
80
100
[θ] (deg·cm²/dmol)
Temperature (°C)
WT
H59L
H59L/H157L

Chapter 4: Binding by the WW and PPIase domains of Pin1: the discovery of two classes of Pin1 interacting proteins

4.1 Introduction

Pin1 is unique amongst all the peptiyl-prolyl isomerases (PPIases) because of its ability to interact with phosphorylated serine or threonine residues followed by a proline (pS/T-P) (*1-4*). Due to this phosphospecificity, Pin1 has been proposed to be a regulatory timer of various cell signalling processes and is found to be involved in many important cellular functions including growth and proliferation, immune response, transcription and apoptosis (*5-8*). Pin1 is also implicated in diseases such as cancer and Alzheimer's disease and has been proposed as a possible therapeutic target (*5, 6, 9*). In spite of the many functional studies and its therapeutic potential, knowledge of Pin1 and its roles in the cell remains incomplete as the molecular mechanisms of Pin1 binding and catalysis are not fully elucidated (*10*). A complete understanding of how Pin1 acts on its targets is needed if we are to fully understand the role Pin1 as a regulator of phosphorylation-dependent processes.

Pin1 is a small, two-domain protein with an N-terminal WW domain and C-terminal catalytic or PPIase domain (*2, 11*). Both domains can recognize pS/T-P motifs (*12, 13*). As the WW domain has a higher binding affinity for peptides than the PPIase domain *in vitro* (*12, 14*), it is thought to act as a protein interaction domain, functioning in protein targeting and enhancing substrate specificity. Similarly, the catalytic function of the PPIase domain has mainly been attributed to its ability to specifically isomerize peptide substrates *in vitro*. It is not known, however, how the two domains of Pin1 act

together on full-length substrates to perform their proper function, or whether all protein targets interact with the two domains of Pin1 in the same manner. Studies have mainly been hampered by the low affinity binding of the PPIase domain and the difficulty in generating full-length phosphorylated substrates *in vitro*.

Many cellular proteins interact with Pin1 (reviewed in (*4, 6*)). These cover a variety of distinct signalling pathways. Interestingly, a number of Pin1 interacting proteins involved in cell cycle progression and mitosis are also antigens of MPM-2 (*3, 13*), an antibody generated against a mitotic lysate and which can recognize phosphoepitopes (*15*). These epitopes include regulators like Cdc25C, Plk1, Wee1 and Myt1, as well as NonO/p54^{nrb} (*12, 13, 16*). Targets may contain single or multiple Pin1 binding sites, and almost all sites have the minimal pS/T-P motif required for interaction. Other sequence or structural factors that determine the binding site on a full-length protein are not known. Recent studies have shown that several targets with multiple Pin1 binding sites, such as Cdc25C, Tau and NonO/p54^{nrb}, have spacing of 18-22 residues between each site suggesting that this may be optimal in an as yet undiscovered mechanism for Pin1 multisite binding (*16, 17*).

Using protein extracts from mitotically-arrested HeLa cells, we employed GST pull-downs to full-length Pin1 and binding-deficient mutations. We found that Pin1 can bind to two different classes of binding proteins: Class I proteins bind to both the WW and PPIase domains while Class II proteins do not have a significant contribution from the PPIase domain to binding. We show that the difference in binding between Class I and Class II proteins is not due to Class II protein abundance. Furthermore, by analyzing known Pin1 binding sites in a Class I interacting protein, we show that this difference can also not be attributed to the number of binding sites. Instead, we suggest that this binding

difference may be due to structural characteristics that induce distinct binding by the two domains of Pin1.

4.2 Methods

Plasmid construction

Constructs for expression of GST, wild-type GST-Pin and GST-Pin1-C113S have been described previously (*18*). Human GFP-C1-Cdc25C was a gift from H. Piwnica-Worms and was used as the template for subsequent Cdc25C cloning. Individual WW and PPIase domains of Pin1 were amplified using the primers listed in Table 4.1, cloned into PCR blunt (Invitrogen) and subcloned using NcoI and HindIII into a pGEX vector. GST-Pin1-R68/69A, and mutations to Pin1 binding sites in Cdc25C were performed using the appropriate primers listed in Table 4.1 and the Quikchange II Site-directed mutagenesis kit (Stratagene), according to manufacturer's instructions. pSPORT6-NonO containing human NonO cDNA was purchased from ATCC. The HA-NonO and HA-Cdc25C plasmids were constructed by PCR using the forward and reverse primers listed in Table 4.1. PCR products were first ligated into PCR blunt (Invitrogen) and screened for the presence of insert before being subcloned into pcDNA3.1-HA_3 (A. French, unpublished results) using XhoI and SalI.

GST fusion protein purification

GST protein expression and purification was performed as described previously (*18*), with bacteria being grown in LB at 37 °C and with overnight induction of protein with 0.6 mM isopropylthio-α-D-β-galactoside at 18 °C. After purification on glutathione-agarose (Sigma) and elution with 10mM reduced glutathione (Sigma), proteins were dialysed into PBS with 20% glycerol overnight at 4°C. The protein concentration after dialysis was determined using the Bradford Protein Assay (Bio-Rad) and proteins were stored at -80 °C until use.

Table 4.1- Oligonucleotides used in site-directed mutagenesis in Chapter 4

Name	Sequence
WW Forward	5'-ATA AGA ATG CGG CCG CCA TGG CGG ACG AGG AGA-3'
WW Reverse	5'-GAA TTC AAG CTT CAG CCG CTG GGC CGC TCC CAC-3'
PPIase Forward	5'-GAA TAT GCG GCC GCC ATG GGG GAG CCT GCC AGG GTC CGC TGC TC-3'
PPIase Reverse	5'-GAA TTC AAG CTT CAC TCA GTG CGG AGG ATG ATG TGG ATG-3'
R68/69A Forward	5'-GAA GCA CAG CCA GTC AGC GGC GCC CTC GTC CTG-3'
R68/69A Reverse	5'-CAG GAC GAG GGC GCC GCT GAC TGG CTG TGC TTC-3'
$p54^{nrb}$ Forward	5'-GGG CTC GAG AAT GCA GAG TCC TAA AAC TTT TAA CTT GGA A-3'
$p54^{nrb}$ Reverse	5'-CAC GTC GAC TTA TTA GTA TCG GCG ACG TTT GTT TGG GG-3'
Cdc25C Forward	5'-CGG TCG ACC ATG TCT ACG GAA CTC TTC TCA TCC-3'
Cdc25C Reverse	5'-CGA CTC GAG TCA TGG GCT CAT GTC CTT CAC CAG AAG GGC-3'
C70R Forward	5'-GAG GAA CCC CAA AAC GTT GCC TCG ATC TTT C-3'
C70R Reverse	5'-GAA AGA TCG AGG CAA CGT TTT GGG GTT CCT C-3'
T48A Forward	5'-GAT GTC CCT AGA GCT CCA GTG GGC AAA TTT C-3'
T48A Reverse	5'-GAA ATT TGC CCA CTG GAG CTC TAG GGA CAT C-3'
T67A Forward	5'-CAT TTT GTC TGG AGG AGC CCC AAA ACG TTG C-3'
T67A Reverse	5'-GCA ACG TTT TGG GGC TCC TCC AGA CAA AAT G-3'
V50P Forward	5'-GTC CCT AGA ACT CCA CCG GGC AAA TTT CTT GGT G-3'
V50P Reverse	5'-CAC CAA GAA ATT TGC CCG GTG GAG TTC TAG GGA C-3'
K69P Forward	5'-CTG GAG GAA CCC CAC CAC GTT GCC TCG ATC TTT C-3'
K69P Reverse	5'-GAA AGA TCG AGG CAA CGT GGT GGG GTT CCT CCA G-3'

Cell culture and transfection

HeLa cells were maintained at 37 °C and 5% CO_2 in DMEM (Invitrogen) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). Transfection of cells at ~80% confluence was performed using the calcium phosphate method with 80 μg of total DNA/15 cm plate (*19*). Transfection efficiency was estimated at 50-70% under these conditions. After 16-18 hours, cells were washed with phosphate buffered saline (PBS; Invitrogen) and fresh media was added. To arrest cells in mitosis, cells were treated with 0.25 μg/mL nocodazole (Sigma) for 18 hours before harvest. Cells were

harvested by collecting cells loosely attached to the tissue culture plate and resuspending them in lysis buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 10% glycerol, 1% Triton X-100) with added protease inhibitors: 1 mM PMSF, 10 μg/mL pepstatin A 1 mg/mL leupeptin, 1 μM microcystin-LR, 1 mM DTT, 1 mM sodium orthovanadate and 1 μM okadaic acid). Cells were allowed to lyse on ice on the bench top for 2-3 hours and cell debris was then spun down by centrifugation first at 13 000 rpm for 15 minutes and then at 55 000 rpm for 30 minutes. Cell lysates were used immediately in pull-downs.

GST pull-downs

For GST pull-downs, 200 μg of GST fusion protein was bound to glutathione cross-linked to agarose beads (Sigma). Beads were washed twice with PBS buffer and once with lysis buffer then incubated with 0.5-2 mg of HeLa lysate for 1 hour at 4 °C. Bound proteins were washed with lysis buffer containing the following protease and phosphatase inhibitors: 1 mM PMSF, 10 μg/mL pepstatin A 1 mg/mL leupeptin, 1 μM microcystin-LR plus 1 mM DTT. After washing 3-5 times, proteins were eluted into SDS sample buffer by boiling. One two-hundredth of the eluted proteins (by volume) of each pull-down was separated, diluted in 20 μL of SDS sample buffer and run on SDS-PAGE before staining with Coomassie Blue to visualize GST fusion protein loading.

Prominent bands on the stained gel were excised with an Ettan Spot Picker (Amersham) and digested using trypsin at the Functional Proteomics Facility (University of Western Ontario). Samples were run on a MALDI-TOF and identified using the MASCOT program.

Western blotting

Pull-downs were run on SDS-PAGE and transferred to polyvinyl difluoride membrane (Millipore). Primary antibodies used include MPM-2 (2 μg/mL; Millipore), Plk1 (1/500; Santa Cruz), Cdc25C (C-20) (1/100; Santa Cruz), SFPQ (1 μg/mL; Abcam), NonO/p54^{nrb} (1 μg/mL; Abcam), HA (12CA5) (1/500; Roche), and gamma tubulin. After incubation with primary antibodies at 4° C overnight, blots were washed and incubated with either GAM or GAR secondary antibodies from Licor Biosciences. Membranes were viewed on a LiCor imager and quantitation was done using Odyssey software (Version 3.0). For quantitation, band intensity on the blot was normalized for the amount of GST fusion protein using the 1/200 Coomassie-stained gel and corrected for the background in the GST lane.

4.3 Results

Pull-downs with Pin1 reveal two different types of binding proteins

Previous studies in the lab generated mutations that were catalytically and structurally deficient (see Chapter 2 and 3, (*20*)). However, we had not yet assessed the effect of specific mutations on the binding of Pin1 to its interaction partners. Accordingly, we used GST fusion proteins encoding wild-type Pin1 or Pin1 mutants to pull down interacting proteins from nocodazole-arrested HeLa lysates. Interestingly, we found that while wild-type Pin1 could pull down many interacting proteins, introducing an R68/69A mutation into Pin1 resulted in a drastic decrease in binding (Figure 4.1A). This result was unexpected as it has been thought that the WW domain of Pin1 with its higher affinity for phosphorylated motifs was responsible for Pin1 interactions, yet the R68/69A mutations lie in the PPIase domain of Pin1. More specifically, these two arginines are part of a large, somewhat flexible loop which has been suggested to help coordinate the phosphate when substrates are bound to the PPIase domain (*2*). By comparison, another mutation in the PPIase domain of Pin1, C113S, which decreases catalytic activity (*21*) displayed a profile similar to wild-type Pin1 (Figure 4.1A). This suggests that the decreased binding seen with R68/69A is not related to isomerase activity, but is due to a binding defect in the PPIase domain.

It is interesting to note that the two most prominent bands in our pull-down, located at ~60 kDa and ~100 kDa bound similarly to all mutations tested including R68/69A. These bands were identified in a wild-type pull-down using MALDI-TOF MS as NonO/p54^{nrb} and its obligate binding partner SFPQ/PSF, respectively (Supplementary Figure 4.1). NonO has previously been shown to interact with Pin1 in a phospho-

dependent manner, although its ability to interact with Pin1 mutants has not been studied (*16*).

To confirm that NonO does indeed bind differently from many other Pin1 interactors, we did small-scale GST pull-downs and evaluated interactions with known Pin1 targets using antibodies. Immunoblotting of pull-downs with MPM-2, an antibody which reacts with a number of Pin1 targets, shows that global binding of interactors is reduced with the R68/69A mutation, except for one band at ~55 kDa. It is extremely likely that the identity of this band is NonO as this protein has been confirmed as an antigen of MPM-2 (*22*). Furthermore, these results are consistent with the pull-down done by Shen *et. al* which showed a reduction in binding of MPM-2 antigens by R68/69A, except for a band at ~55 kDa (*13*).

Looking more specifically at the known Pin1 targets Plk1 and Cdc25C, we found that these proteins follow the same pattern as seen with the MPM-2 antibody, with less binding to R68/69A compared to wild-type (Figure 4.1B). Interestingly, pull-downs with C113S appear to slightly increase interaction (Figure 4.1C). This may be because the kinetics of this mutation shows that its low isomerase activity is due to a decrease in k_{cat} while its K_M increases slightly (*20*). These results show that for the majority of Pin1 interactors, the presence of an intact PPIase domain can increase binding to a target significantly, especially in the case of Cdc25C where binding with R68/69A is undetectable. This is surprising given that the WW domain has more than ten times greater affinity for Pin1 targets than the PPIase domain. For example, Lu *et. al* demonstrated that the optimal Pintide substrate (WFYpSPFLE) had K_ds of 1.2μM and 11μM for the WW and PPIase domain, respectively (*12*). However, using pull-downs,

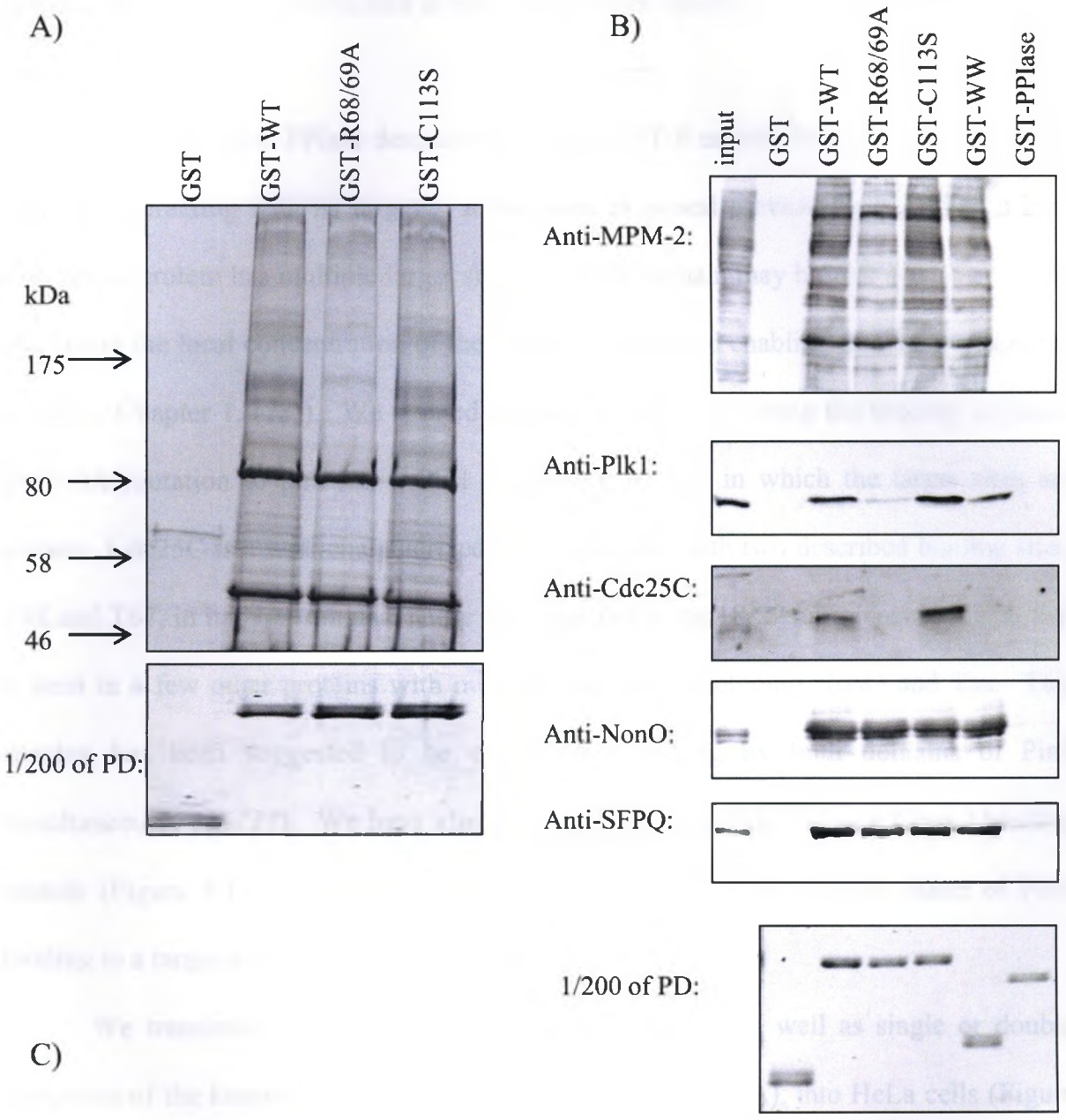
we did find that the PPIase domain did not bind to any targets on its own, whereas the WW domain could (Figure 4.1B).

Using antibodies, we verified that NonO bound equally well in wild-type Pin1 and R68/69A pull-downs. This suggests that NonO does not rely on the PPIase domain significantly for binding and that there may be at least two different classes of Pin1 proteins, ones that are bound by both the WW and PPIase domains (which we have called Class I proteins) and ones where the PPIase domain does not contribute as much to binding (named Class II proteins). To our knowledge, this is the first study to suggest that Pin1 does not bind to all of its interacting proteins in the same way. Knowing how Pin1 binds is necessary to understand its role in cells.

NonO binding to R68/69A is not due to protein abundance

It is obvious from our GST pull-downs in Figure 4.1A that NonO and SFPQ/PSF represent the most abundant Pin1 interacting proteins. As these were also the proteins which were pulled out in equal amounts in wild-type and mutant Pin1 pull-downs, we wanted to ensure that this effect was not due to their abundance. Therefore, pull-downs with Pin1 mutants were performed and serial dilutions of the pull-down run on a Western Blot. Lower serial dilutions did not show any difference between R68/69A and wild-type with respect to their interactions with NonO (Figure 4.2A). NonO levels were also the same when a lower amount of lysate was used in the pull-downs (Figure 4.2B). We then exogenously expressed NonO at lower levels than the endogenous protein (Figure 4.2C) and compared levels of both HA-NonO and HA-Cdc25C in Pin1 pull-downs on the same blot. We found that, like their endogenous counterparts, NonO still bound R68/69A while Cdc25C did not (Figure 4.2D). These results confirm that binding of NonO to Pin1-R68/69A is not simply due to its abundance.

Figure 4.1- GST pull-downs with Pin1 mutants reveal two different types of binding proteins. A) Large-scale GST pull-downs. One to two milligrams of nocodazole-treated HeLa lysates were incubated with GST fusion proteins bound to glutathione beads. Interacting proteins were run on a 5-12% gradient gel and stained with Coomassie Blue. B) GST pull-downs were performed as in A) with 1mg of HeLa lysate. Interacting proteins were run on 10% SDS-PAGE, transferred to PVDF and blotted with the indicated antibody. To better compare the amount of fusion protein on the beads, 1/200 of each pull-down was run on a gel and stained with Coomassie Blue. C) Quantification of blots shown in Figure 1B). Results are the mean of three independent pull-down experiments ± S.E.M.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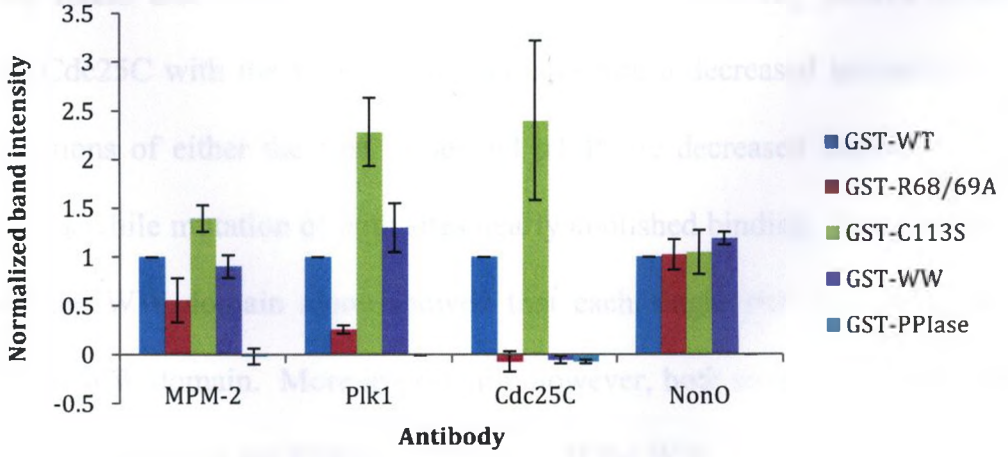
GST
GST-WT
GST-R68/69A
GST-C113S
kDa
175
80
58
46
1/200 of PD:
B)
input
GST
GST-WT
GST-R68/69A
GST-C113S
GST-WW
GST-PPIase
Anti-MPM-2:
Anti-Plk1:
Anti-Cdc25C:
Anti-NonO:
Anti-SFPQ:
1/200 of PD:

C)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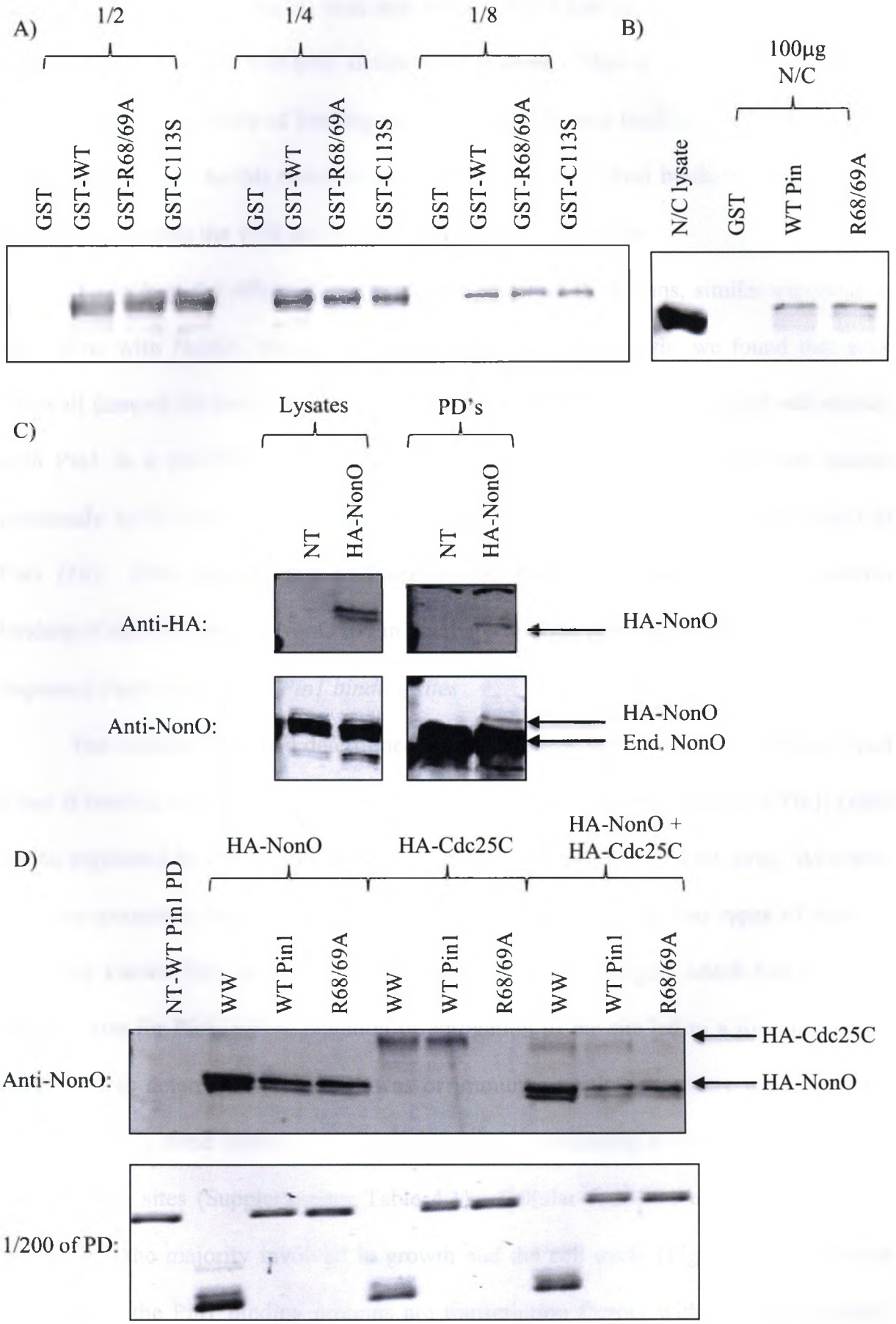
3
2.5
2
1.5
1
0.5
0
-0.5
MPM-2
Plk1
Cdc25C
NonO
Antibody
GST-WT
GST-R68/69A
GST-C113S
GST-WW
GST-PPIase

Binding of R68/69A to interactors is not related to the number of available Pin1 binding sites

As its WW and PPIase domain both target pS/T-P motifs, Pin1 may have multiple ways of interacting with its targets. It has been proposed previously that when a Pin1 interacting protein has multiple target sites, the WW domain may bind at one site thereby increasing the local concentration of the PPIase domain and enabling it to bind at another site (See Chapter 1, (*23*)). We wanted to test this theory by using the binding deficient R68/69A mutation to pull out a Pin1 interacting protein in which the target sites are known. Cdc25C is a well-characterized Pin1 interactor with two described binding sites, T48 and T67, in its N-terminus. These two sites fulfill the 18-22 amino acid spacing that is seen in a few other proteins with multiple Pin1 sites including NonO and Tau. This spacing has been suggested to be optimal for binding by both domains of Pin1 simultaneously (*16, 17*). We have also previously defined Cdc25C as a Class I binding protein (Figure 4.1). For these reasons, we chose Cdc25C to test the effect of Pin1 binding to a target with single or multiple sites.

We transiently transfected wild-type GFP-Cdc25C, as well as single or double mutations of the known Pin1 binding sites (T48A and/or T67A), into HeLa cells (Figure 4.3A). We found that GFP-Cdc25C followed the same binding pattern to Pin1 as endogenous Cdc25C with the R68/69A mutant showing a decreased interaction (Figure 4.3B). Mutations of either the first or second pT-P site decreased binding to Pin1 by more than 50% while mutation of both sites nearly abolished binding. Furthermore, pull-downs with the WW domain alone showed that each single site had about the same affinity for the WW domain. More importantly however, both single mutations show the same decreased binding to the R68/69A mutation. If the WW domain was binding at one

Figure 4.2- p54^{nrb}/NonO binding to R68/69A is not due to protein abundance. A) GST pull-downs were performed as in Figure 1A) and serial dilutions of the pull-down were run on a gel as indicated and immunoblotted with anti-p54^{nrb}/NonO. B) Pull-downs were performed as in Figure 1A, but with 100 μg of HeLa lysate. C) HeLa cells were either transfected with HA-NonO or left untransfected (NT). Lysates were used in GST pull-downs as in Figure 1A) and immunoblotted with the indicated antibody. D) HeLa cells were transfected with HA-NonO or HA-Cdc25C or left untransfected (NT). Lysates were used in pull-downs as in Figure 1A) and transfected proteins were detected with anti-HA. For the combined sample, 1mg of HA-NonO lysate was mixed with 1mg of HA-Cdc25C lysate.


A)
1/2
1/4
1/8
GST
GST-WT
GST-R68/69A
GST-C113S
B)
100µg
N/C
N/C lysate
GST
WT Pin
R68/69A
C)
Lysates
PD's
NT
HA-NonO
Anti-HA:
HA-NonO
Anti-NonO:
HA-NonO
End. NonO
D)
HA-NonO
HA-Cdc25C
HA-NonO +
HA-Cdc25C
NT-WT Pin1 PD
WW
WT Pin1
R68/69A
Anti-NonO:
HA-Cdc25C
HA-NonO
1/200 of PD:

site and the PPIase to another, then one would expect that an interactor with only one target site should bind R68/69A similar to wild-type. This is because only the WW domain would be capable of binding and the PPIase domain binding defect of R68/69A would not be seen. As this is not the case, we conclude that Pin1 binds to targets such as Cdc25C by binding the WW and PPIase domains on a single site.

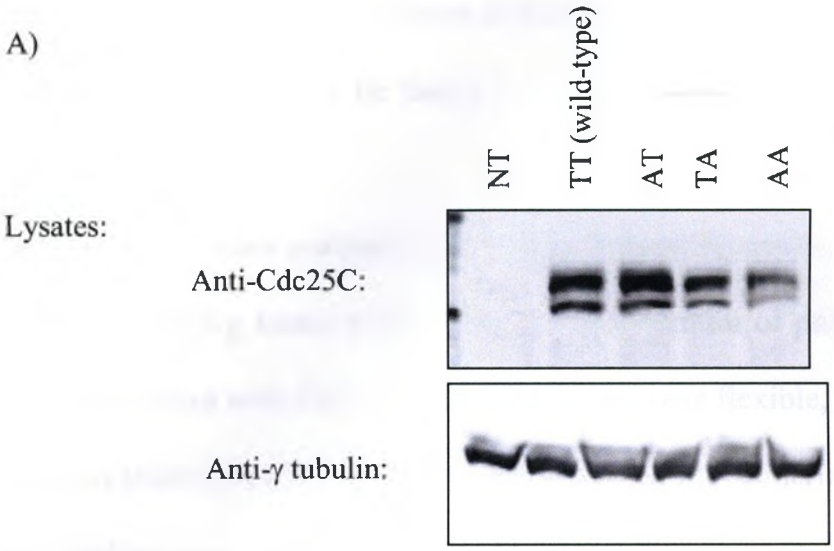
To look at the effect of multiple sites on Class II proteins, similar experiments were done with NonO. However, despite extensive mutagenesis, we found that even when all three of the binding sites in NonO were mutated to alanine, it could still interact with Pin1 in a pull-down (Supplementary Figure 4.2). These sites had been shown previously to be responsible for direct binding of mitotically phosphorylated NonO to Pin1 (*16*). This suggests that pull-downs with NonO were complicated by indirect binding of mutant forms of NonO to Pin1 perhaps through its binding partner SFPQ/PSF.

Sequence characteristics of Pin1 binding sites

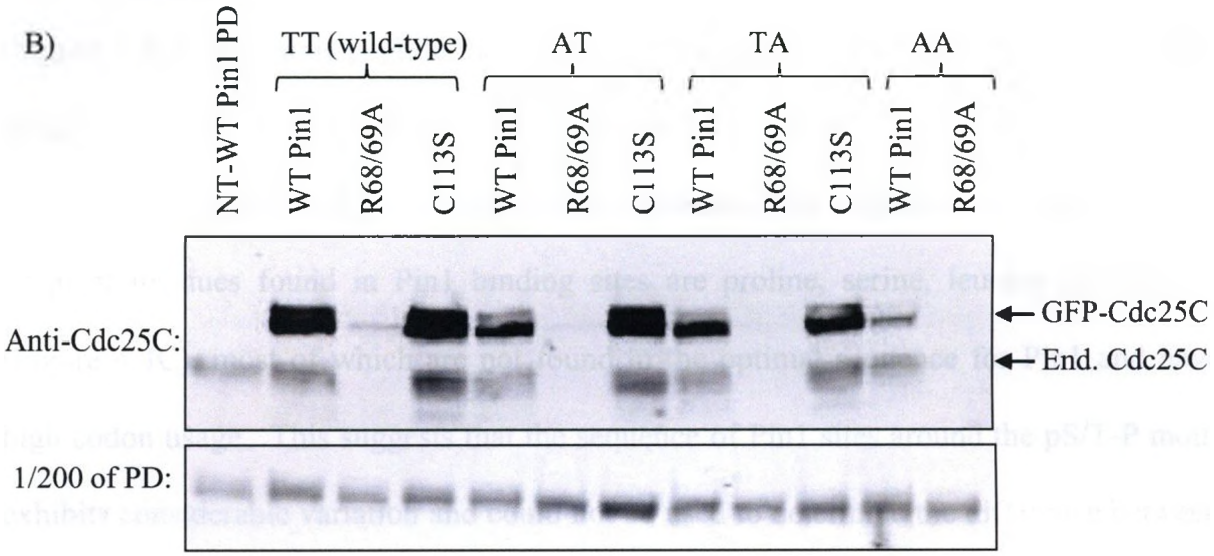
Our previous data had determined that differences in binding between Class I and Class II binding sites, which differ in their binding by the PPIase domain of Pin1, could not be explained by differences in abundance or by the number of Pin1 sites. As a next step, we examined possible sequence differences between these two types of sites by analysing known Pin1 targets in the literature. Only Pin1 targets which had definitive binding sites for Pin1, where mutation or abrogation of the site led to a loss of physical interaction as determined by pull-downs or immunoprecipitations, were used in further analysis. With these criteria, we found 39 proteins containing a cumulative total of 58 Pin1 binding sites (Supplementary Table 4.1). Cellular functions of these 39 proteins varied with the majority involved in growth and the cell cycle (Figure 4.4A). Almost one-third of the Pin1 binding proteins are transcription factors with the most common

Figure 4.3- Binding of R68/69A to interactors is not related to the number of available Pin1 binding sites. A) HeLa cells were either not transfected (NT) or transfected with GFP-Cdc25C containing the following mutations: TT (wild-type Cdc25C), AT (T48A), TA (T67A), AA (T48/67A). Lysates from transfections were run and immunoblotted with the Cdc25C antibody. γ-tubulin was used as a loading control. B) and C) Lysates from A) were used in pull-downs with B) GST-Pin1 or C) GST-WW fusion proteins. To better compare the amount of fusion protein on the beads, 1/200 of each pull-down was run on a gel and stained with Coomassie Blue.

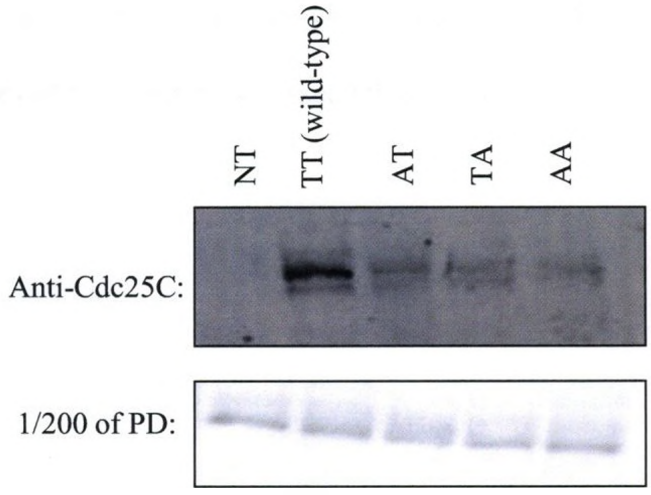
A)


NT
TT (wild-type)
AT
TA
AA
Lysates:
Anti-Cdc25C:
Anti-γ tubulin:

B)


NT-WT Pin1 PD
TT (wild-type)
AT
TA
AA
WT Pin1
R68/69A
C113S
WT Pin1
R68/69A
C113S
WT Pin1
R68/69A
C113S
WT Pin1
R68/69A
Anti-Cdc25C:
GFP-Cdc25C
End. Cdc25C
1/200 of PD:

C)

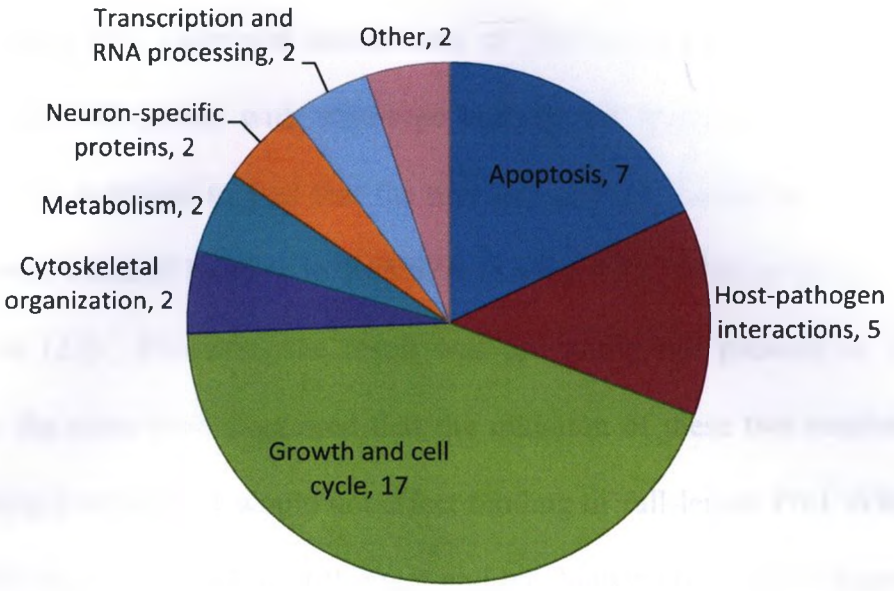

NT
TT (wild-type)
AT
TA
AA
Anti-Cdc25C:
1/200 of PD:

effect of Pin1 being the stabilization or destabilization of the target. Furthermore, most of the proteins analysed can be found in the nucleus (Figure 4.4B) consistent with Pin1 localization (*1*).

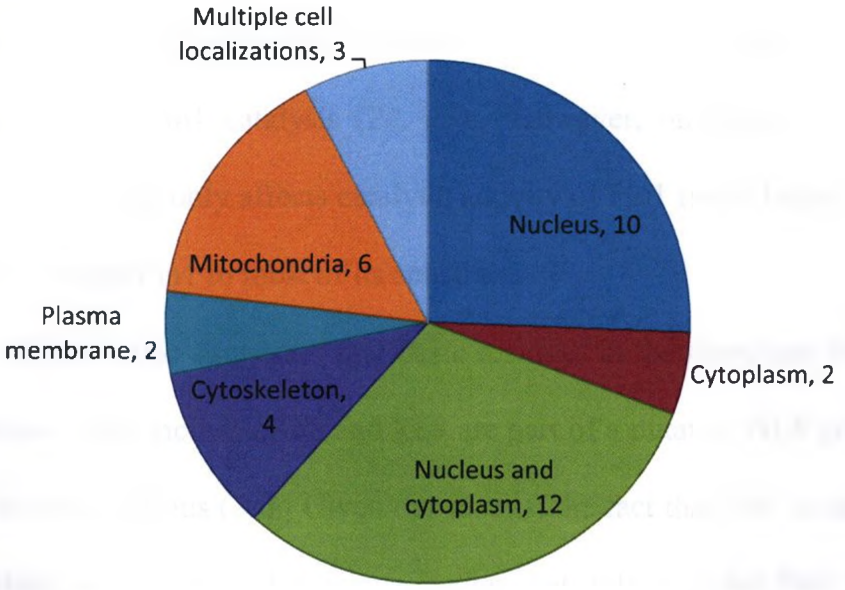
In the 58 sites analysed, only ~12% of them lay within known structural domains with the rest being found either at the N- or C-termini of proteins or between domains. This is consistent with Pin1 preferring to act on more flexible, unstructured regions of the protein to allow space for the 180° rotation needed for isomerization (*10*). Analysis of the Pin1 binding sites showed considerable variability at positions around the S/T-P motif (Figure 4.4C). Previous peptide mapping had identified the optimal Pin1 sequence to be highly aromatic (W/F/Y-F/I-Y/R/F/W-pS-P-R/F/Y/W-L/I) (*3*). However, none of the Pin1 sites identified in the literature thus far contains this sequence. In fact, the most frequent residues found in Pin1 binding sites are proline, serine, leucine and alanine (Figure 4.4C), most of which are not found in the optimal sequence for Pin1 and have high codon usage. This suggests that the sequence of Pin1 sites around the pS/T-P motif exhibits considerable variation and could not be used to determine the difference between our two classes of binding sites. Instead, we propose that the differences in binding by the PPIase domain, which distinguishes Class I and Class II interacting proteins, is due to structural features of the binding site (see Discussion).

Figure 4.4- Analysis of known Pin1 binding sites. A) Distribution of the functions of the 39 Pin1 interacting proteins analysed. B) Distributions of the localization of the Pin1 interacting proteins analysed. C) A frequency graph of known Pin1 binding sites from the literature. Amino acids at the top of the graph show the highest frequency. Diagram was generated using WebLogo.

A)


Transcription and RNA processing, 2
Other, 2
Neuron-specific proteins, 2
Apoptosis, 7
Metabolism, 2
Cytoskeletal organization, 2
Host-pathogen interactions, 5
Growth and cell cycle, 17

B)


Multiple cell localizations, 3
Nucleus, 10
Mitochondria, 6
Plasma membrane, 2
Cytoplasm, 2
Cytoskeleton, 4
Nucleus and cytoplasm, 12

C)


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
N
C

4.4 Discussion

In this study, we examined interactions of full-length Pin1 with targets from mitotic cells using pull-downs with wild-type and mutant forms of GST-Pin1 fusion proteins. We were surprised to find that the majority of Pin1 interacting proteins from these cells showed reduced binding to R68/69A (Figure 4.1). This result is consistent with Shen *et. al* (*13*). However, the result was previously not pursued as structural studies done at the same time suggested that the mutation of these two residues in the phosphate-binding loop of Pin1 would not affect binding of full-length Pin1 to substrates because the WW domain, which is still intact and has higher affinity for targets, would still function (*2*). In fact, other studies have used the R68/69A mutation to test whether their phenotype requires Pin1 catalysis (*24, 25*). However, our study would caution against this as R68/69A not only affects catalytic activity of Pin1 (see Chapter 2), but also the binding of full-length Pin1 to most of its substrates.

Lufei *et al.* have also suggested that basic residues in the phosphate binding loop of the PPIase domain that includes R68 and R69 are part of a putative NLS governing the import of Pin1 into the nucleus (*26*). Given our results, the fact that Pin1 is small enough to enter the nucleus on its own and previous studies that indicated that Pin1 localization was mediated mainly through binding of its targets (*1, 13*), it seems more likely that the R68/69A mutation decreases its nuclear localization because of reduced target interaction rather than causing a defect in nuclear import. Accordingly, we propose that one should not assume when using the R68/69A mutation in studies that it will only affect PPIase activity as it also often loses its affinity for the target protein. Instead, our results indicate that R68/69A may be useful in determining the contribution of the PPIase domain to

substrate binding whereas a mutation like C113S which has been shown to have decreased catalytic activity (see Chapter 2) (*21*), but does not affect binding to substrates (Figure 4.1) would be preferable as a catalytically deficient mutant.

The other surprising result from our pull-downs is that the R68/69A mutation did not decrease binding to NonO, suggesting that its interaction with Pin1 is mainly through the WW domain (Figure 4.1). Although this protein, along with its binding partner SFPQ, were the only proteins we found to do this in our study, the original characterization of the interaction of the microtubule-associated protein Tau with Pin1 demonstrated that Tau phosphorylated on T231 bound equally to wild-type Pin1 and a K63A mutant, a residue which, like R68 and R69, is involved in phosphate binding in the PPIase domain (*2, 20, 27*). NMR studies with phosphorylated peptides showed that pT231 could bind the WW but not the PPIase domain of Pin1 while a Tau peptide phosphorylated at T212 (another binding site for Pin1) was able to bind both domains (*14, 17*). These studies suggest that the T231 site on Tau may be another Class II binding site.

Sequence characteristics of Pin1 binding sites themselves, beyond the minimal pS/T-P motif, remain ill-defined. As a first step in determining features that define a Pin1 binding site, we examined 58 known Pin1 binding sites from the literature. Alignment of these known Pin1 sites reveals no distinguishing sequence features (Figure 4.4C) although an optimal binding sequence for full-length Pin1 had previously been determined (*3*). One possibility for why the optimal sequence differs from natural Pin1 binding sites may be because these natural sequences have selection pressures involving other signalling proteins. Additional selection pressures could include kinases that originally phosphorylate the site as well as phosphatases, E3 ubiquitin ligases, and other

protein interactors that may act on this site. Pin1 may even have developed very minimal sequence requirements to accommodate for the specificities of other enzymes working in the substrate-specific pathway. Pin1 sites also seem to be in more unstructured regions of the protein, perhaps to allow for the conformational changes that isomerization incurs, and so it is likely that stretches of secondary structure-inducing residues are not as common in Pin1 sites.

Alternatively, as we had determined that the difference between Class I and Class II binding sites was due to differences in binding to the PPIase domain, we focused on how structures of the WW and PPIase domains bind to phosphopeptides. Although the two domains of Pin1 bind to the same pS/T-P sequence, structures of these domains with peptides or peptide inhibitors show that they do not do so in the same way. Peptides bound to the WW domain are extended, with little bend induced in the peptide backbone (*28*) (Figure 4.5A). The only structures of the PPIase domain bound to a longer peptide are those with a PPIase specific peptide inhibitor (*29*). The backbone of the peptides in these structures form a type I β-turn in the active site of Pin1. This places the carbonyl oxygen of the -1 residue and the amino hydrogen of the +1 residue close enough together to form an intramolecular hydrogen bond. However, if the +1 residue in the Pin1 site was a proline, then there would not be an amino hydrogen at the +1 position to form this bond. This may alter the affinity of the peptide for the PPIase domain or, alternatively, may increase the isomerization of the substrate as the hydrogen bond would not have to be broken or extended to allow for the conformational change to occur. In either case, a +1 positioned proline would cause less binding by the PPIase domain while the affinity of the WW domain remained the same. Notably, this +1 proline could be used as an

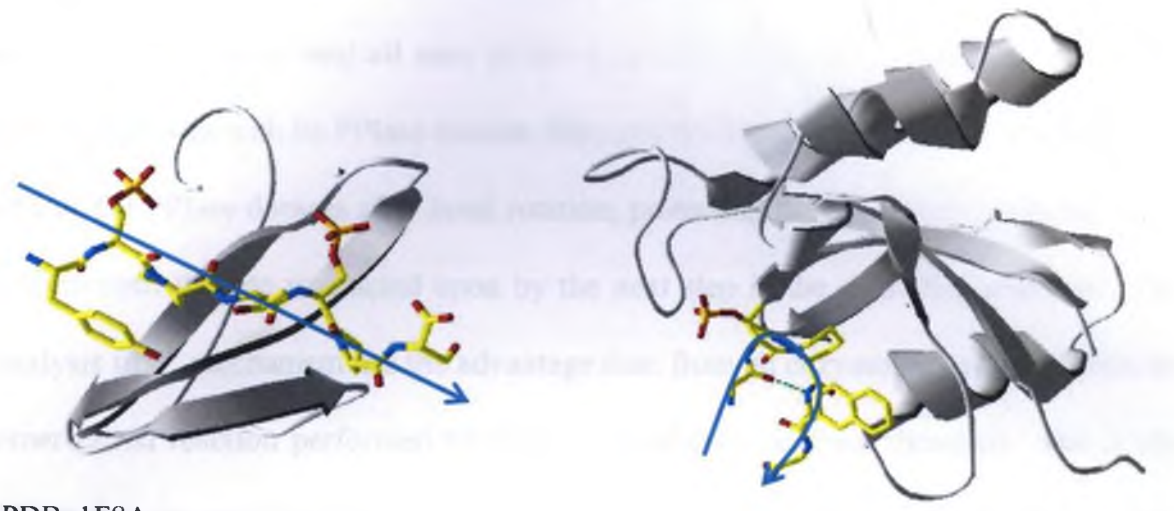
explanation for the difference in Class I and II binding sites as our Class II interacting protein NonO has two binding sites where the +1 residue is a proline (Figure 4.5B). This would indicate that, unlike Class I binding sites which bind both Pin1 domains, the two sites in NonO with a +1 proline would not bind to the PPIase domain distinguishing it as a Class II binding protein. It is interesting that the T231 site on Tau also contains a +1 proline which would be consistent with our proposed model that Class I and Class II interacting proteins differ structurally because of the residue in their +1 position. In summary, this model will be useful in further characterization of the sequence and/or structural differences in Class I and Class II binding proteins.

One question that remains unanswered is the possible role these Class II binding sites play in the cell. The function of the Pin1 binding sites in NonO is not known, but it may be that binding mainly of the WW domain of Pin1 to these sites allows them to act as "sinks", regulating active Pin1 by sequestering it away from potential substrates until it is needed. The large abundance of NonO in the cell lends support to this idea and it is possible that the decrease of NonO in ER-negative breast cancer may be one way of deregulating Pin1 in disease (*30*).

Alternatively, the difference between Class I and II interactors may relate to how the WW and PPIase domain perform Pin1 functions together. It has been proposed that full-length Pin1 is first targeted to a site via its WW domain and the PPIase follows acting on either the same site (in a sequential binding mechanism) or on a proximal site (in a simultaneous binding mechanism) similar to the targeting of other modular proteins with specific protein interaction domains. In this case, PPIase binding sites would have specific features (such as those suggested in Figure 4.5) which would increase or decrease the interaction of the PPIase domain with the protein depending on the class of interactor.

Figure 4.5- Phosphopeptide binding by the WW and PPIase domains. A) Structures of the WW domain (left) and PPIase domain (right) of Pin1 bound to peptides or peptide inhibitors. A general trace of the backbone of the peptide is shown in blue. B) Sequences of sites in the two Pin1 binding proteins used in this study, Cdc25C and NonO, with pT-P and +1 residues highlighted.

A)



PDB: 1F8A

PDB: 2ITK

B)

Cdc25C		NonO	
Site 1(T48):	DVPRpTPVGKF	Site 1(T410):	VPAGpTPAPPG
Site 2 (T67):	LSGGpTPKRCL	Site 2 (T428):	TLGLpTPPTTE
		Site 3 (T450):	AIGGpTPPAFN

Another mechanism of binding proposed early in the study of the Pin1 was that the WW domain, which was shown to be specific for the *trans* form of the T48 binding site in Cdc25C, would bind all sites in this form (*31*). Any remaining Pin1 would then target the *cis* sites with its PPIase domain first, and the higher affinity WW domain would displace the PPIase domain after bond rotation, protecting the bond from returning to the *cis* form until the site was acted upon by the next step in the signalling pathway. This "catalysis first" mechanism has the advantage that, from an enzymology view, it gives the isomerization reaction performed by Pin1 a specific *cis* to *trans* direction. But it also requires that the enzyme work at near stoichiometric levels in the cell, even though Pin1 has been shown to work at substoichiometric levels on Cdc25C (*32*). Still, if this mechanism were to hold true, the difference between Class I and II interacting proteins could be explained by differences in the amount of *cis* and *trans* isomers of each site generated in the cell. It would also mean that Pin1 likely evolved to keep S/T-P sites which would spontaneously generate *cis* isomer populations upon phosphorylation, in the *trans* form so that they could be acted upon by their *trans*-specific enzymes.

Regardless of which binding mechanism occurs in cells, the separation of Pin1 interacting proteins into two classes is important as each class will respond differently to any potential Pin1 therapeutics which target either the WW or the PPIase domain specifically.

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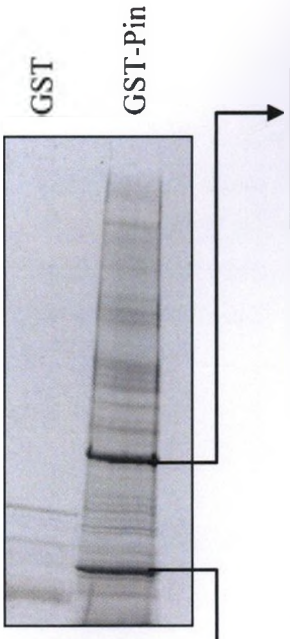
Supplementary Figure 4.1- Identification of SFPQ and NonO in GST-Pin1 pull-downs. The ~55 kDa and 100 kDa bands were excised from the gel and subjected to trypsin digest. Samples were run on a MALDI-TOF and identified using the MASCOT program. Sequence coverage for each protein is shown in red.



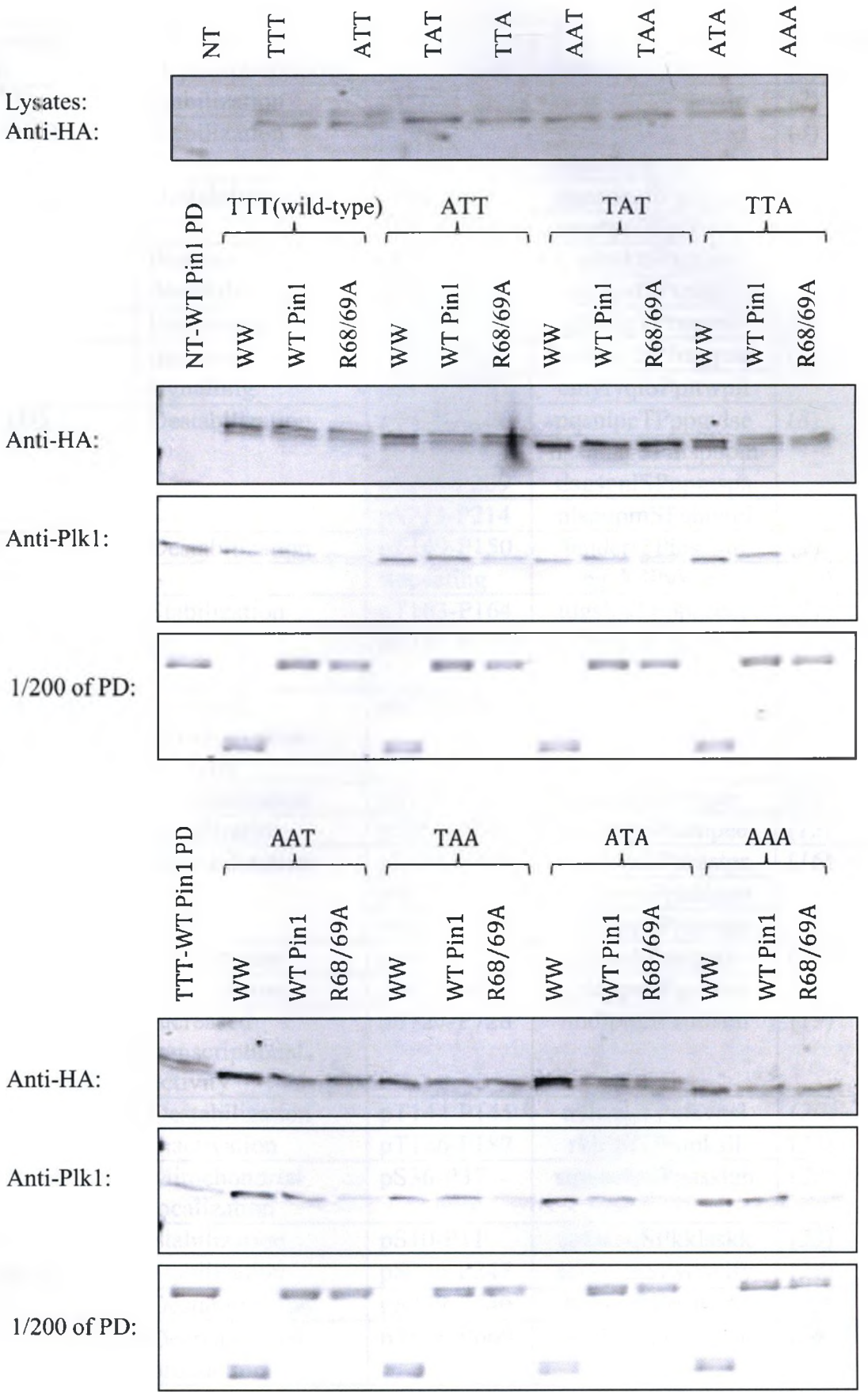
SFPQ: 31.0% Coverage

MSRDRFRSRGGGGGGFHRRGGGGGRGGLHDFRSPPPGMGLNQNRGP
MGPGPGQSGPKPPIPPPPPHQQQQQPPPQQPPPQQPPPHQPPPHPQ
PHQQQQPPPPPQDSSKPVVAQGPGPAPGVGSAPPASSSAPPATPPT
SGAPPGSGPGPTPTPPPAVTSAPPGAPPPTPPSSGVPTTPPQAGGP
PPPPAAVPGPGPGPKQGPGPGGPKGGKMPGGPKPGGGPGLSTPGGH
PKPPHRGGGEPRGGRQHHPPYHQQHHQGPPPGGPGGRSEEKISDSE
GFKANLSLLRRPGEKTYTQRCRLFVGNLPADITEDEFKRLFAKYGE
PGEVFINKGKGFGFIKLESRALAEIAKAELDDTPMRGRQLRVRFAT
HAAALSVRNLSPYVSNELLEEAFSQFGPIERAVVIVDDRGRSTGKG
IVEFASKPAARKAFERCSEGVFLLTTTPRPVIVEPLEQLDDEDGLP
EKLAQKNPMYQKERETPPRFAQHGTFEYEYSQRWKSLDEMEKQQRE
QVEKNMKDAKDKLESEMEDAYHEHQANLLRQDLMRRQEELRRMEEL
HNQEMQKRKEMQLRQEEERRRREEEMMIRQREMEEQMRRQREESYS
RMGYMDPRERDMRMGGGGAMNMGDPYGSGGQKFPPLGGGGGIGYEA
NPGVPPATMSGSMMGSDMRTERFGQGGAGPVGGQGPRGMGPGTPAG
YGRGREEYEGPNKKPRF

NonO: 48.6% Coverage

MQSNKTFNLEKQNHTPRKHHQHHHQQQHHQQQQQQPPPPPIPANGQ
QASSQNEGLTIDLKNFRKPGEKTFTQRSRLFVGNLPPDITEEEMRK
LFEKYGKAGEVFIHKDKGFGFIRLETRTLAEIAKVELDNMPLRGKQ
LRVRFACHSASLTVRNLPQYVSNELLEEAFSVFGQVERAVVIVDDR
GRPSGKGIVEFSGKPAARKALDRCSEGSFLLTTFPRPVTVEPMDQL
DDEEGLPEKLVIKNQQFHKEREQPPRFAQPGSFEYEYAMRWKALIE
MEKQQQDQVDRNIKEARFKLEMEMEAARHEHQVMLMRQDLMRRQEE
LRRMEELHNQEVQKRKQLELRQEEERRRREEEMRRQQEEMMRRQQE
GFKGTFPDAREQEIRMGQMAMGGAMGINNRGAMPPAPVPAGTPAPP
GPATMMPDGTLGLTPPTTERFGQAATMEGIGAIGGTPPAFNRAAPG
AEFAPNKRRRY

Supplementary Figure 4.2-Binding of NonO and its mutations to Pin1. HeLa cells were either not transfected (NT) or transfected with HA-NonO containing combinations of mutations to its three binding sites, T410, T428, T450. Lysates from transfections were run and immunoblotted with the 12CA5 antibody. g-tubulin was used as a loading control. Lysates were used in pull-downs with GST-Pin1 fusion proteins. To better compare the amount of fusion protein on the beads, 1/200 of each pull-down was run on a gel and stained with Coomassie Blue.


NT
TTT
ATT
TAT
TTA
AAT
TAA
ATA
AAA
Lysates:
Anti-HA:
NT-WT Pin1 PD
TTT(wild-type)
ATT
TAT
TTA
WW
WT Pin1
R68/69A
Anti-HA:
Anti-Plk1:
1/200 of PD:
TTT-WT Pin1 PD
AAT
TAA
ATA
AAA
Anti-HA:
Anti-Plk1:
1/200 of PD:

Supplementary Table 4.1- Known Pin1 Binding Sites

Interactor	Effect of Pin1	Pin1 site	Pin1 site sequence	Reference
FAK	Dephosphorylation	pS910-P911	klqpqeiSPppptanld	(*1*)
$p27^{Kip1}$	Stabilization	pT187-P188	nagsveqTPkkpglrr	(*2*)
Akt/PKB	Stabilization	pT92-P93	ertfhveTPeereewt	(*3*)
		pT450-P451	taqmitiTPpdqddsm	
SULT4A1	Destabilization	pT8-P9	maeseaeTPsptpgef	(*4*)
		pT11-P12	seaetpsTPgefesky	
Btk	Protein destabilization	pS21-P22	sqqkkktSPlnfkkrl	(*5*)
		pS115-P116	egplyvfSPteelrkr	
Bax	Prosurvival	pT167-P168	gllsyfgTPtwgtvti	(*6*)
Tax	Increased NFκB signalling	pS116-P117	lqamrkySPfrngyme	(*7*)
		pS160-P161	cmylyqlSPpitwpll	
SMAD3	Destabilization	pT179-P180	pqsnipeTPppgylse	(*8*)
		pS204-P205	ngsndagSPnlspnpm	
		pS208-P209	dagspnlSPnpmspa	
		pS213-P214	nlspnpmSPahnnld	
TRF1	Destabilization	pT149-P150	fenderiTPlesalmi	(*9*)
NF-H		Repeating	eg. kSPvkeea	(*10*)
MCl	Stabilization	pT163-P164	tdgslpsTPppaeeee	(*11*)
CTP synthase 1		pS575-P576	sdrsgssSPdgeitel	(*12*)
c-Myb	Increased transactivation acitivity	pS528-P529	kikqeveSPtdksgnf	(*13*)
Daxx	Destabilization	pS178-P179	aentasqSPrtrgsrr	(*14*)
Bcl6	Stabilization	pS260-P261	vchsniySPketipee	(*15*)
PML	Destabilization	pS403-P404	aavskkaSPeaastpr	(*16*)
		pS518-P519	pstskavSPphldgpp	
		pS527-P528	phldgppSPrspvigs	
HBx	Stabilization	pS41-P42	lgtlsspSPsarpadh	(*17*)
Bim_{EL}	Stabilization	pS65-P66	gplappaSPgpfatrs	(*18*)
Stat3	Increased transcriptional activity	pS727-P728	ntidlpmSPrtldslm	(*19*)
Che1	Destabilization	pT144-P145	trshsakTPgfsvqsi	(*20*)
Wee1	Inactivation	pT186-P187	rklrlfdTPhtpksll	(*21*)
$p66^{shc}$	Mitochondrial localization	pS36-P37	stppeelpSPpasslgp	(*22*)
Emi1	Stabilization	pS10-P11	cgfasnqSPkklsskk	(*23*)
β-catnenin	Stabilization	pS246-P247	alvkmlgSPvdsvlfy	(*24*)
IRF3	Destabilization	pS339-P340	ftegsgrSPryalwfc	(*25*)
APP	Decreased Aβ production	pT668-P669	vevdaavTPeerhlsk	(*26, 27*)

Synphilin-1	Increased interaction with α-synuclein	pS211-P212	nmapfcvlSPvksphlr	(*28*)
		pS215-P216	cvlspvkSPhlrkasa	
c-Fos	Increased transcriptional response	pT232-P233	gglpevaTPeseeaft	(*29*)
p53	Stabilization	pS33-P34	lpennvlSPlpsqamd	(*30-33*)
		pS46-P47	amddlnlSPddieqwf	
		pT81-P82	apapaapTPaapapap	
		pS315-P316	lpnntssSPqpkkkp	
P54^{nrb}/NonO		pT410-P411	papvpagTPappgpat	(*34*)
		pT428-P429	pdgtlglTPptterfg	
		pT450-P451	gigaiggTPpafnraa	
RARα	Destabilization	pS77-P78	eivpsppSPpplpriy	(*35*)
c-Myc	Destabilization	pT58-P59	kkfellpTPplspsrr	(*36*)
Tau	Dephosphorylation	pT212-P213	tpgsrsrTPslptppt	(*37, 38*)
		pT231-P232	kkvavvrTPpkspssa	
P65/RelA of NFκB	Stabilization	pT254-P255	qvaivfrTPpyadpsl	(*39*)
Bcl2		pS70-P71	rdpvartSPlqtpaap	(*40*)
		pS87-P88	aaagpalSPvppvvhl	
Cdc25C	Dephosphorylation	pT48-P49	vcdvprTPvgkflgd	(*37, 41, 42*)
		pT67-P68	lsilsggTPkrcldls	
RPB1	CTD dephosphorylation	Repeating	[ysptSPs]	(*43-46*)
Cyclin D1	Stabilization	pT286-P287	eevdlacTPtdvrdvd	(*47*)
c-Jun	Transactivation	pS63-P64	knsdlltSPdvgllkl	(*48*)
		pS73-P74	vgllklaSPelerlii	

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Chapter 5: Summary and Perspectives

5.1 Introduction

The PPIase Pin1 is involved in the regulation of many diverse cellular pathways and also has potential as a therapeutic target in cancer (reviewed in (*1-3*)). However, in spite of being structurally well-characterized, the molecular mechanisms of Pin1 action remain ill-defined. Mechanisms for isomerization and models for binding by the WW and PPIase domains of Pin1 have been presented, but our understanding is incomplete (*4*). It is not even known whether isomerization by Pin1 is necessary for cell function although conservation of PPIase activity in parvulins of diverse organisms imply that this is likely (*5-9*). In an effort to better understand the actions of Pin1, my studies were directed towards testing the hypothesis that Pin1 acts as an extra regulatory step in signalling pathways by first binding to phospho-specific targets with its WW domain and then catalyzing the *cis* to *trans* isomerization of phosphomotifs in those same proteins using its PPIase domain. To examine this hypothesis, we evaluated the isomerase activity, interactions with targets and function of specific residues in Pin1 that had been identified in a previous unbiased random mutagenesis strategy (*10*). Our studies revealed several new insights into the catalytic mechanism of Pin1 and with the development of our new non-covalent catalytic model, we speculate that all three groups of PPIases may use similar mechanisms for isomerization to decrease the barrier of rotation. In addition, our examination of Pin1 binding to Cdc25C supports two of the three previously reported binding models for Pin1, and our results imply that Pin1 binding may change depending on the characteristics of the interdomain linker and the cellular levels of Pin1.

5.2 Catalysis by Pin1

As a PPIase, Pin1 is able to catalyze rotation around the peptide bond preceding a proline (*7*). However, Pin1 enzymes also have the additional unique property that allows them to participate in cell signalling: their ability to specifically recognize and isomerize phosphorylated residues preceding the proline (*11*). Indeed, analysis has shown that phosphorylation enhances the efficiency of catalysis by more than 1000-fold (*12*). As it has been shown that addition of a phosphate can decrease the rate of non-catalytic isomerization quite significantly (*13*), it is likely that Pin1 has been specifically adapted to perform catalysis of pS/T-P motifs at a rate which matches the time scale required in cellular reactions. In line with this, although PPIases differ in their substrate specificity, the Pin1 enzymes have the highest catalytic efficiencies of all the Cyclophilins (CyP's), FK506-binding proteins (FKBP's) and parvulins measured thus far (*14*).

The original crystal structure of Pin1 led to a proposed mechanism of covalent catalysis with roles for the trio of residues H59, C113, and H157 based on their position in the active site (*11*). Interpretation of the structure also implicated that the three basic residues in the loop between the first β-sheet and the first α-helix of the PPIase domain had roles in binding the phosphate moiety of the substrate. Although initial alanine substitutions at these positions did support the original model (*11*), the covalent mechanism for catalysis has since come under scrutiny as sequences and structures of other non-Pin1 parvulins with high sequence similarity reveal that they have an aspartic acid at the catalytic C113 position (*15-17*). Our mutations to this position reveal that Pin1 does use a non-covalent mechanism for catalysis similar to other PPIases.

5.2.1 Conclusions from mutational analysis

To build on our earlier unigenic evolution analysis that identified regions of Pin1 that were needed for function (*10*), we examined the roles of individual residues in these regions for function in our plasmid shuffling assay and for *in vitro* isomerase activity (Table 5.1). Our data revealed several new aspects on how Pin1 performs its catalytic function and suggested a possible model for a non-covalent mechanism for isomerization by bond distortion.

a) Role of the substrate phosphate

The original crystal structure of Pin1 had a sulphate moiety in the active site which was shown to be coordinated by three basic residues, K63, R68 and R69 in the loop between the first β-sheet and the first α-helix of the PPIase domain (Figure 2.4A) (*11*). It was proposed that these three residues may similarly coordinate the phosphate of the Pin1 substrate although it did not look at the contributions of each residue. To further examine how Pin1 binds the phosphate in its PPIase domain, we mutated each of these basic residues to alanine and tested their function in our yeast plasmid shuffling assay (Figure 2.4B). We found that of the three residues, only a single alanine mutation to K63 failed to support viability in yeast while single mutations to R68 or R69 were functional. Consistent with previous research (*18*), a double alanine substitution of these two basic residues was able to disrupt function although its isomerase activity was not as low as the K63A mutation (Figure 2.4B). These results suggest that all of these three residues are involved in coordination of the phosphate, but to different extents. Based on the mutational data, K63 appears to be the most important residue for phosphate binding, with one other contribution from either R68 or R69.

Table 5.1- Summary of mutations

Mutation	Functionality in yeast	Isomerase activity (% of WT)[a)]	Binding activity
WT	+	100	+
Y23F	+	N.D.	N.D
Y23A	-	N.D.	-[b)]
W34R	+	N.D.	N.D.
R68A	+	N.D.	N.D.
R69A	+	N.D.	N.D.
R69W	+	N.D.	N.D.
R68/69A	-	25.0[c)]	+/-[d)]
K63A	-	3.6[c)]	N.D.
C113D	+	28.6	N.D.
C113S	-	1.2	+
C113N	-	N.D.	N.D.
H59A/N/S	+	29.1-49.8	N.D.
H59L/F	-	0[e)]-3.3	+[f)]
H157L/A/N/F/S	+	5.9-82.8	+[f)]
H59L/H157L/A/F	+	5.0-41.2	+[f)]
H59L/H157N/S	-	0.57-4.9	N.D.

N.D.= not determined
a) Activity measured with Pintide (WFYpSPR-*p*NA) substrate unless indicated
b) Data not shown.
c) Activity measure with Suc-AEPF-*p*NA
d) Majority of interactions were decreased
e) No detectable activity when stock concentrations of protein used
f) Binding tested only for the leucine mutation

Interestingly, although we were not looking at phosphate binding in Chapter 3, we did find that *in vitro* activity of a set of histidine mutations we were working with had a different rank order depending on the substrate used (Figure 3.3C). The phosphorylated WFYpSPR (Pintide) substrate showed a much better correlation with function *in vivo* compared to the unphosphorylated AEPF substrate. Both substrates had been used in the literature previously (*11-13*), as it was anticipated that the glutamic acid in the unphosphorylated substrate would act similarly to the phosphate of Pintide. However, our results show that this is not the case, and suggest that the phosphate group itself may be involved in the catalysis.

b) Role of H59 and H157

Our initial interest in the two conserved histidines in the active site of Pin1, H59 and H157 (described in Chapter 2), arose from the discovery that mutation of H59 to Q was functional in yeast (Figure 2.5B). This was an unexpected result given the high conservation of this residue in parvulins as well as its important proposed role in the original nucleophilic attack catalytic mechanism model (*11*). An H59L mutation was not functional, however, suggesting that there may be a requirement for hydrogen bonding at this position. Notably, mutations made to H157 did not affect viability in yeast at all.

In Chapter 3, we extended our studies on these two histidine residues after it was serendipitously discovered that a second mutation of H157 to L could rescue the non-functional H59L (Figure 3.2). This prompted us to make various mutations to both H59 and H157 and test them for both functionality in yeast and activity in our isomerase assay. Surprisingly, we found that many mutations to H59 and all mutations to H157 were functional in yeast (Figure 3.1). We also found that mutating H157 to hydrophobic residues could rescue the inviable H59L (Figure 3.2). Isomerase activities of the histidine

mutants were similar to those in yeast with substitution of either H59 or H157 by small amino acids (such as A or N) being less detrimental than large, hydrophobic mutations (such as L or F). However, the low isomerase activity of H59L could be rescued by a second hydrophobic mutation to H157. Collectively, these results suggested that H59 and H157 were connected. Furthermore, comparison of isomerase activity of mutanats in H59 and H157 to that of the C113S mutation suggested that these two conserved residues did not play as prominent a role in the catalytic mechanism as C113.

To provide a better view of the role of H59 and H157, we further examined our histidine mutants using partial proteolysis and circular dichroism (CD). We found that mutations to H59 and H157 were more susceptible to trypsin proteolysis than wild-type Pin1 (Figure 3.4) and destabilized at lower temperatures as analyzed by following CD signal (Figure 3.6). Importantly, in both cases the second mutation of H157 to L partially rescued the destabilizing effect of the H59L mutation. Consequently, instead of acting as catalytic acids and bases as was proposed in the original isomerase mechanism of Pin1, our results point to a structural role for H59 and H157 in the Pin1 active site.

c) Role of C113

The mutations made to H59 and H157 in Chapter 2 prompted us to re-evaluate the role of the catalytic residue C113, originally proposed to act as a nucleophile and attack the carbonyl carbon of the prolyl bond. We mutated this residue to both S and D to test if Pin1 prefers a nucleophile or a negative residue at this position. Surprisingly, we found that as opposed to C113S, the C113D mutation was functional in yeast and had higher isomerase activity than the inviable C113S (Figure 2.5). This confirms that C113 does not play a nucleophilic role in catalysis, but may need to be negative. This would explain why other parvulin enzymes have an aspartic acid at the C113 position (*15-17*). Our

results in Chapter 3, confirmed that C113 is more important for catalysis than the two histidines in the active site.

5.2.2 A new catalytic model for Pin1

Results from our mutational analysis allowed us to propose a novel non-covalent mechanism for isomerization (Figure 2.6B). This mechanism includes a negative charge at position C113 to decrease the double bond character of the peptide bond, allowing rotation. We suggested that it is hydrogen bonding in the unique environment of the Pin1 active site that allows the sulfhydryl group of C113 to present a partial or full negative charge to the carbonyl oxygen of the substrate. Although our results in Chapter 3 suggest that it is not H59 that makes the major hydrogen bond to C113, other residues are still within hydrogen bonding distance and pKa predictions made using the PROPKA program (*19*) do suggest that C113 has a negative charge at physiological pH (Figure 2.6C).

Although representative high resolution structures have been elucidated for each of the three groups of PPIases, none of the CyPs, FKBPs or parvulins has a validated catalytic mechanism (*14*). It is interesting that our general bond distortion mechanism, where the barrier to rotation is lowered due to the stabilization of the more apolar tetrahedral form of the bond, can be applied to the other groups of PPIases. As they share a similar fold, the active site of Pin1 can be overlaid on FKBP12 (Figure 5.1B). Comparisons of the active sites reveals that not only do H59 and H157 of Pin1 correspond to Y26 and F99 of FKBP12 (as shown in Figure 3.8), but the C113 position overlays well with an aspartic acid (D37) in FKBP12. Mutation of this residue in other studies does decrease catalytic activity *in vitro* (*20, 21*) which suggests that D37 may be the negative charge needed for bond distortion by FKBP12.

Comparison of the active sites of Pin1 and CyPs is more difficult as they do not share the same fold. However, overlays of the active sites based on bound peptides does not show any close negative charges to the carbonyl oxygen of the peptide bond, but does show a positive charge (R55 in CyP18) which lies within hydrogen bonding distance to the imide group of the proline (Figure 5.1A). Mutation of this residue in CyP18 does decrease isomerization activity significantly (*22*) and it may be that in CyPs, a positive charge presented to the proline imide has a similar effect of bond distortion as a negative charge presented to the carbonyl oxygen. In this way, all of the PPIases may use this same general mechanism for bond distortion to aid catalytic rotation as part of their overall catalytic mechanism.

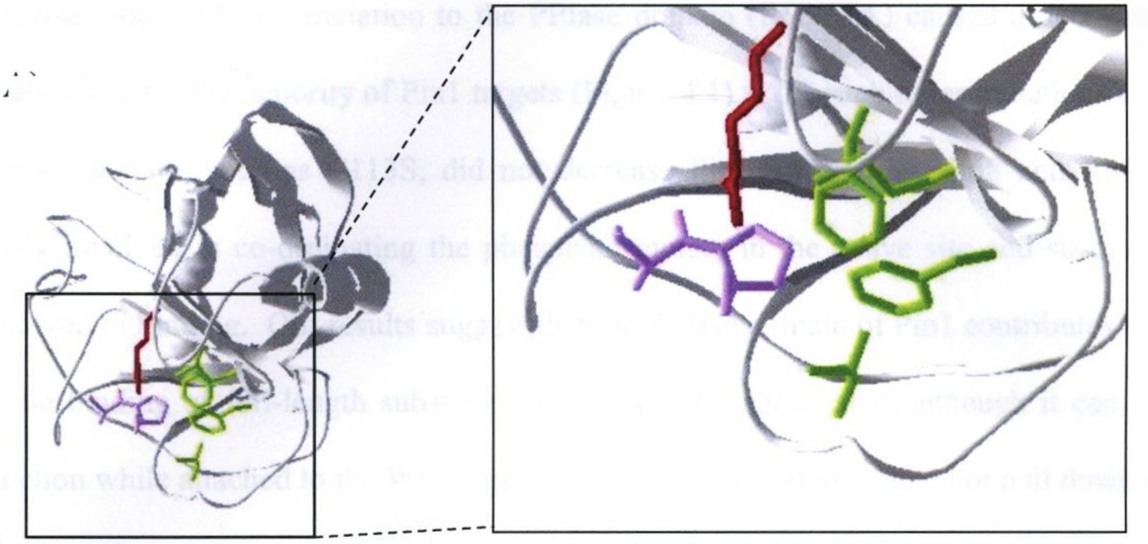
5.3 Binding by Pin1

It is known that both the WW and PPIase domains of Pin1 can bind to pS/T-P motifs (*11, 12, 23, 24*). Furthermore, the WW has defined as a targeting domain as it has at least 10- to 20-fold higher affinity for targets than the PPIase domain (*23, 25*). It has, however, been difficult to assess the binding of both domains together to full-length phosphorylated targets because of the complexity of obtaining phosphoproteins *in vitro*. Instead, we used the mutations generated previously in our studies to study two-domain binding by Pin1.

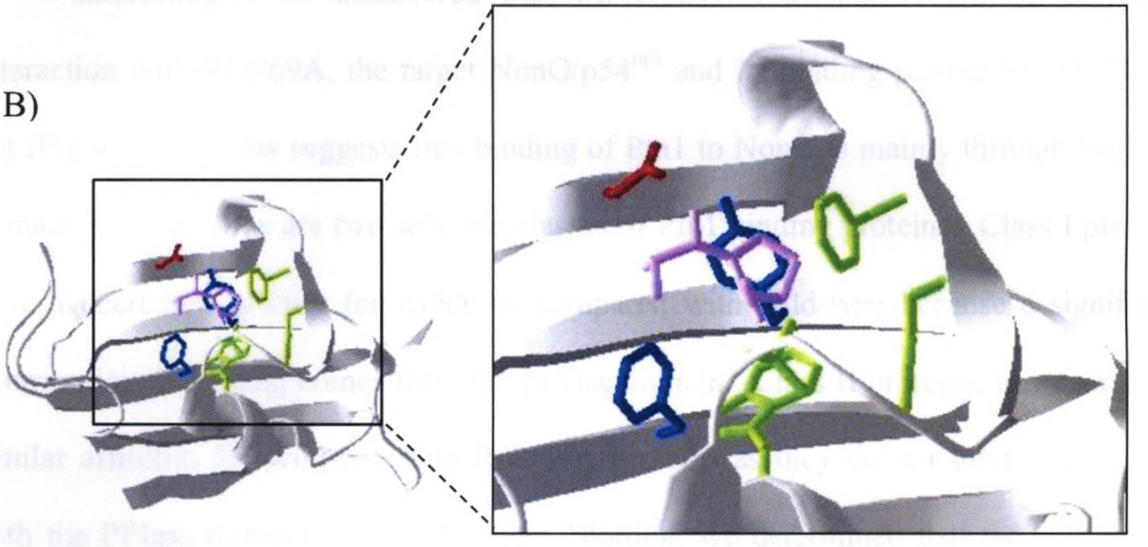
5.3.1 Conclusions from mutational analysis

In Chapter 4, we looked at the effects of Pin1 mutations on binding to targets using pull-down assays. Given the theories in the literature (*4, 12, 23*) and the results of pull-downs with H59 and H157 mutations in Figure 3.7, we anticipated that Pin1 interactions would mainly be mediated by the high affinity WW domain. We were

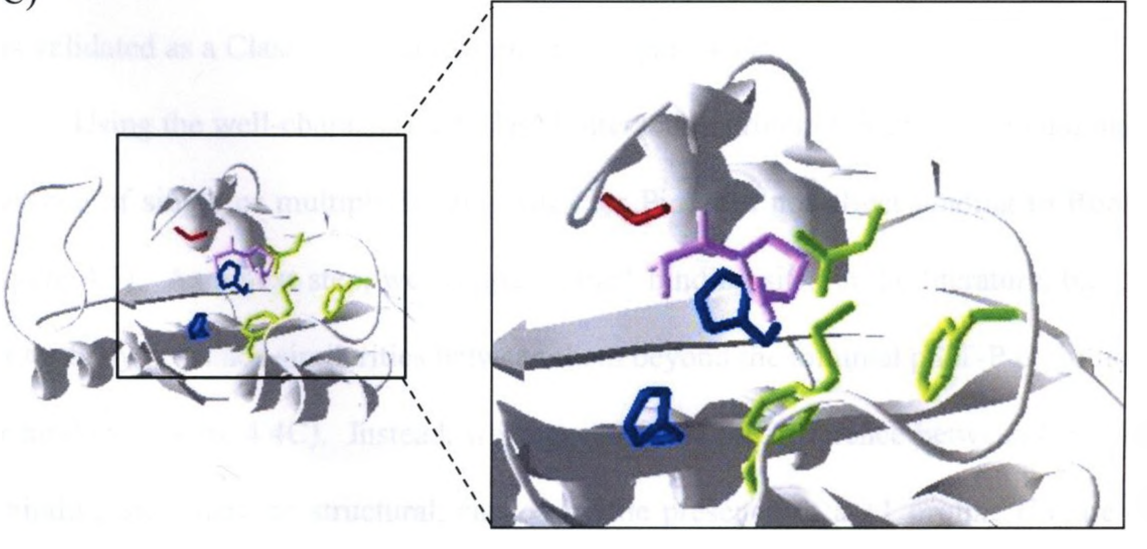
Figure 5.1- A comparison of the active sites of A) Cyp18, B) FKBP12 and C) the PPIase domain of Pin1. An Ala-Pro dipeptide which was part of the crystal structures of Cyp18 and Pin1 and which was placed in the active site of FKBP12 based upon structural alignment with Pin1 is shown in pink. Residues which spatially correspond to H59 and H157 in Pin1 are shown in blue. Suggested residues forming the proline binding pocket are shown in green. Possible important catalytic residues for bond distortion (R55 in Cyp18, D37 in FKBP12 and C113 in Pin1) are shown in red.



B)



C)



surprised then, when a mutation to the PPIase domain (R68/69A) caused a decrease in interactions for the majority of Pin1 targets (Figure 4.1). Although other mutations in the PPIase domain, such as C113S, did not decrease Pin1 interactions, R68 and R69 are likely involved in co-ordinating the phosphate moiety in the active site and so directly involved in binding. Our results suggest that the PPIase domain of Pin1 contributes more to the binding of full-length substrates than originally anticipated, although it can only function while attached to the WW domain as the PPIase domain could not pull down any targets on its own.

Interestingly, we discovered that while most Pin1 targets exhibit decreased interaction with R68/69A, the target NonO/p54^{nrb} and its binding partner SFPQ/PSF do not (Figure 4.1). This suggests that binding of Pin1 to NonO is mainly through the WW domain and that there are two separate classes of Pin1 binding proteins. Class I proteins have a decreased affinity for R68/69A compared with wild-type because a significant contribution to binding comes from the PPIase domain. Class II proteins, however, have similar affinities for wild-type and R68/69A proteins as they do not bind significantly with the PPIase domain. Using Western Blotting, we determined that the two known Pin1 interacting proteins Cdc25C and Plk1 were Class I interacting proteins while NonO was validated as a Class II interacting protein (Figure 4.1).

Using the well-characterized Class I interacting protein Cdc25C, we found that the presence of single or multiple binding sites for Pin1 did not affect binding to R68/69A (Figure 4.3). As a next step, we compared Pin1 binding sites in the literature, but could not find any sequence similarities between them beyond the minimal pS/T-P motif needed for binding (Figure 4.4C). Instead, we suggested that the difference between Class I and II binding sites may be structural, caused by the presence of a +1 proline (Figure 4.5).

Results from Chapter 4 represent important new findings for Pin1 as the presence of two types of Pin1 binding sites has not yet been presented in the literature.

5.3.2 Models for Pin1 binding to Cdc25C

In the introductory chapter of this thesis (Figure 1.4), we presented three possible modes of binding for full-length Pin1: the simultaneous model, the sequential model and the catalysis first model. In Chapter 4, we looked at the binding of Cdc25C and Pin1 using various mutations in each of the two proteins. Consistent with previous research (*18, 23, 26*), we found that Pin1 bound mainly to T48 and T67 in the regulatory N-terminus of Cdc25C (Figure 4.3). Additionally, we found that the PPIase domain of Pin1 contributes significantly to the binding of full-length Cdc25C by full-length Pin1 even if only one of the two binding sites is available. Although our data do not provide conclusive evidence for binding to Cdc25C by one of the three models listed above, the data for each model can now be evaluated.

The binding of the WW and PPIase domains to two distinct sites at the same time, termed the simultaneous model, is one possible model for Pin1-Cdc25C binding. But it is inconsistent with our data in Chapter 4 as Cdc25C with a mutation of one of the two binding sites (either T48 or T67) still had a lower affinity for R68/69A compared with wild-type Pin1. If the simultaneous model were responsible, then the defect of a binding-deficient PPIase mutation like R68/69A would be minimal on a substrate with a single site because binding would occur exclusively through the higher affinity WW domain. There is still the possibility, however, that the PPIase domain may bind another site in Cdc25C or in another Cdc25C interacting partner. Accordingly, our data in Chapter 4 indicate that the PPIase domain binding deficient R68/69A mutation may be a useful tool

in determining sites of PPIase binding, more so than using the PPIase alone which has a very low affinity for targets.

The sequential model, where the WW domain targets a binding site and the PPIase domain follows and binds to that same site, is consistent with our evidence from Chapter 4 where a single Pin1 binding site on Cdc25C would still be affected by mutations that decrease the binding contribution from the PPIase domain. However, other studies have suggested that the WW domain binds to T48 of Cdc25C in *trans* (*27*). It would then follow that the PPIase domain acting on the same site would catalyze the *trans* to *cis* isomerization of that site which is inconsistent with the role Pin1 has been shown to play in promoting dephosphorylation of Cdc25C by the *trans*-specific phosphatase PP2A (*18*). In addition, it would be difficult for the lower affinity PPIase domain to displace the WW on the target sequence. Therefore, our evidence in Chapter 4 does support the sequential model. However, data from other studies suggest that this is not how Pin1 binds to Cdc25C in cells.

The final model, the catalysis first binding model, where all of the *trans* forms of the binding site are bound by the WW domain leaving the *cis* forms to be catalyzed to *trans* by the PPIase domain also fits well with our data from Chapter 4. Furthermore, this model has the advantage of explaining the difference in binding between Class I and Class II binding sites. Class II binding sites, like those in NonO for example, would not be affected by defective PPIase binding because there would be a high proportion of prolyl bonds in the *trans* conformation which bind only to the WW domain. The disadvantage of this model is that Pin1 must work at near stoichiometric levels on Cdc25C which is inconsistent with a previous study which found that Pin1 can catalyze a conformational change in Cdc25C at substoichiometric levels (*28*).

Among the three prevailing models for Pin1 binding, the data from this thesis best supports either the sequential or catalysis first models for binding to the two known binding sites on Cdc25C although binding to other target sites should still be examined. Additionally, the catalysis first model was able to explain the difference we saw between Class I and II binding sites. In general, given that no one binding model can explain Pin1 action exclusively, it is likely that binding by the two domains of Pin1 may depend on other factors such as the nature of the interdomain linker, which can vary in different organisms, or the levels of Pin1 in the cell.

The linker between the WW and PPIase domains of Pin1 is not well-conserved between organisms and can be short but flexible, as in the human Pin1 homologue, or long and structured, as with fungal homologues (Figure 5.2A). It has been suggested that distinct linkers may allow the WW and PPIase domains to interact differently on a substrate. For example, the structured linker in the crystal structure of Ess1 from *C. albicans* brings the two domains close enough together so that both the WW and PPIase domains could bind a substrate like the RNA polymerase II CTD at the same time (Figure 5.2B). The domains of human Pin1, however, have little contact with each other in solution because of the more flexible linker which may broaden the specificity for human substrates by allowing alternative modes for binding.

Variations of Pin1 levels may also change the Pin1 binding mechanism depending on whether Pin1 is at stoichiometric levels to its target in the cell. Although the human Pin1 promoter is not fully mapped, it is known to be an E2F target and transcript and protein levels of Pin1 have been shown to increase in the G1/S part of the cell cycle in non-neoplastic cells (*29*). This pathway is often deregulated in cancer which can account for the overexpression of Pin1 seen in many cancers (*30-33*). Furthermore, the Pin1

Figure 5.2- The interdomain linker of fungal and human Pin1. A) Sequence alignment of human Pin1, Ess1 from *S. cerevisiae*, and Ess1 from *C. albicans* is shown. Secondary structure elements are shown for the *C. albicans* structure where elements in the WW domain are red, the linker region is yellow and those in the PPIase domain are green. Structural elements agree well with the human Pin1 structure with the exception of the α-helix in the linker region. B) Crystal structures of *C. albicans* Ess1 and human Pin1 are shown along with an NMR structure of full-length human Pin1. Domain colour scheme is the same as in A). WW domains are all aligned in the same orientation to show the different orientations of the PPIase domain between Pin1 and Ess1. Peptides which were originally crstallized in two separate structures of human Pin1 (an RNA polymerase CTD peptide in the WW domain and a peptide inhibitor in the PPIase domain) are in each of the structures and the N and C termini of each are indicated. Note that part of the flexible linker is missing in the human Pin1 crystal structure and the NMR structure is only one representative structure.

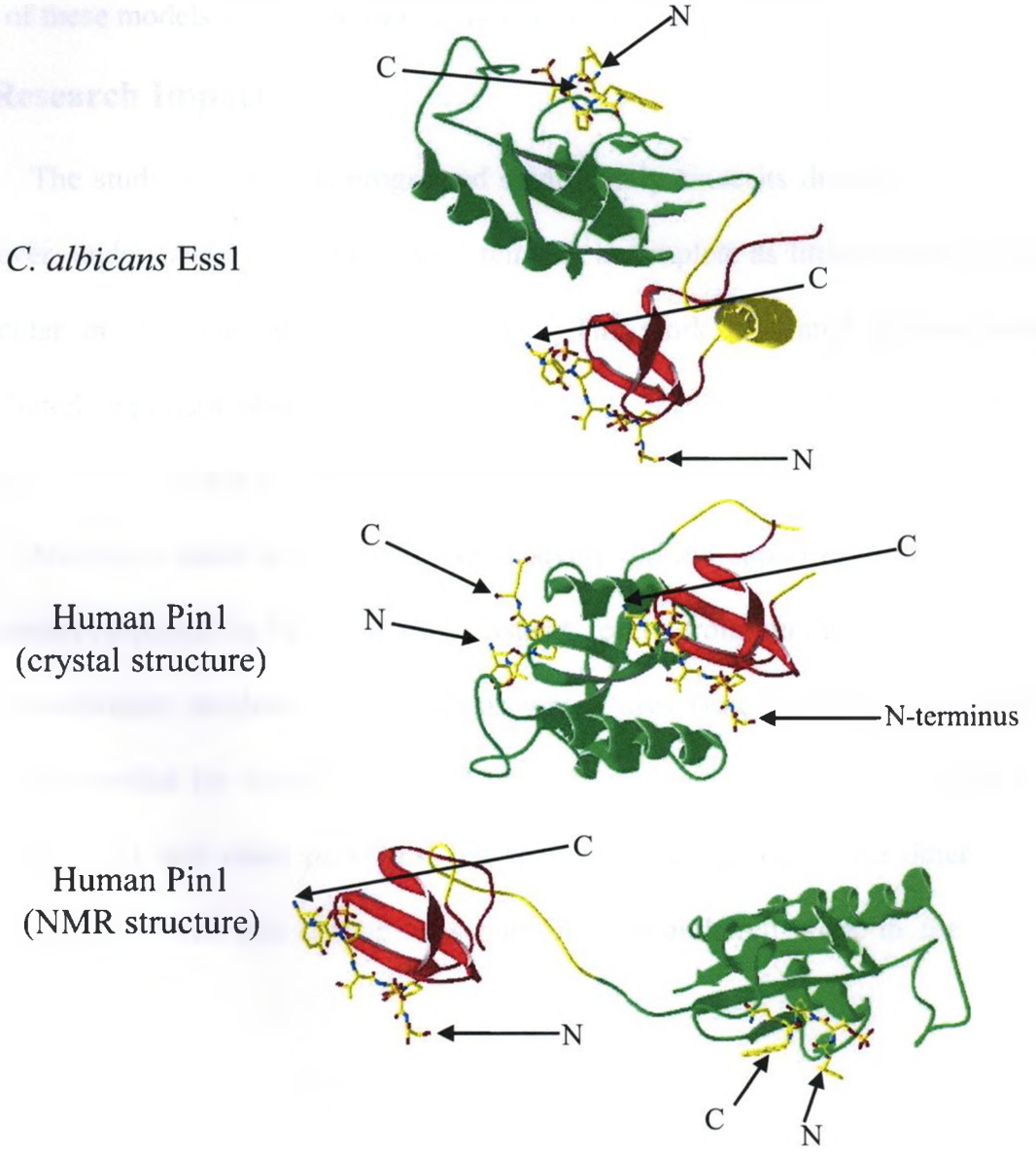
A)

```
Human Pin1 ----MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSG-NSSSG----------GKN
S. cer Ess1  MPSDVASRTGLPTPWTVRYSKSKKREYFFNPETKHSQWEEPEGTNKDQL----------HKH
C. alb Ess1  ---MASTSTGLPPNWTIRVSRSHNKEYFLNQSTNESSWDPPYGTDKEVLNAYIAKFKNNGYKP
```

```
Human Pin1 GQGEPARVRCSHLLVKHSQSRRPSSWRQ-EKITRTKEEALELINGYIQKIK--SGEEDFESLA
S. cer Ess1  LRDHPVRVRCLHILIKHKDSRRPASHRS-ENITISKQDATDELKTLITRLDDDSKTNSFEALA
C. alb Ess1  LVNEDGQVRVSHLLIKNNQSRKPKSWKSPDGISRTRDESIQILKKHLERIL--SGEVKLSELA
```

```
Human Pin1 SQFSDCSSAKARGDLGAFSRGQMQKPFEDASFALRTGEMSGPVFTDSGIHIILRTE
S. cer Ess1  KERSDCSSYKRGGDLGWFGRGEMQPSFEDAAFQLKVGEVSDIVESGSGVHVIKRVG
C. alb Ess1  NTESDCSSHDRGGDLGFFSKGQMQPPFEEAAFNLHVGEVSNIIETNSGVHILQRTG
```

B)



promoter has been shown to have rare polymorphisms that are linked with Alzheimer's disease as well as several putative neuron-specific elements which may account for the high expression of Pin1 in brain tissue (*34*). As alterations in Pin1 expression have been correlated with pathogenesis, it is essential to know whether increased expression changes the mechanism of binding of Pin1 to one that works at stoichiometric levels, such as the catalysis first model.

In summary, simultaneous, sequential and catalysis first binding by full-length Pin1 may all occur in various organisms, and future studies on Pin1 should consider all three of these models when determining how Pin1 may interact with a specific target.

5.4 Research Impact

The study of Pin1 has progressed significantly since its discoverey in 1989 (*5*). However, understanding of this enzyme remains incomplete as little is known about the molecular mechanisms of actions of Pin1. The work presented in this thesis has contributed important observations regarding Pin1 functions. Significantly, data from Chapters 2 and 3 of this thesis have already been published.

Mutations made in Chapter 2 conclusively did not support the original covalent mechanism proposed for Pin1 catalysis. Instead, results from our data led us to propose a new non-covalent mechanism of catalysis which uses bond distortion to decrease the barrier of rotation for isomerization. This new mechanism represents a good testable model for Pin1 and other parvulins, but may also be applied to the other groups of PPIases. Data from this chapter was part of a co-authored work in the Journal of Molecular Biology.

This work was extended in Chapter 3, where we discovered that two absolutely conserved histidines, H59 and H157, were more important for the structure of the PPIase domain rather than catalysis itself. This study put forth a new role for these two histidines and suggests that they are not crucial in the new catalytic mechanism for Pin1. These results were published in Biochemistry.

Finally, we used mutations generated in Chapter 2 to investigate the binding of Pin1 to targets and found that the PPIase domain of Pin1 contributes significantly to the binding of most full-length targets. Furthermore, our studies showed that there are at least two different types of Pin1 interacting proteins, which to our knowledge represents a novel observation about the binding of Pin1. A literature search of known Pin1 binding sites showed that these sites did not have distinguishing sequence characteristics and may be recognized on the basis of their structure. Together, these data emphasize that little amount that is known about the binding mechanisms of the WW and PPIase domains to targets, knowledge which would be valuable in developing therapeutics. These results, presented in Chapter 4, are currently being prepared for submission.

5.5 Future Directions

a) Analysis of other Pin1-target interactions

Our investigation of Pin1 interacting proteins yielded the interesting observation that there are two classes of Pin1 binding proteins. Although we went on to characterize one example in each of the two classes further, more data from other interacting proteins would help define the structural or sequence determinants that separate these two classes. Another example of a Class II interacting protein would be especially useful, as results from our Class II binding protein, NonO, were complicated by its likely indirect binding

to Pin1. One possibility for a Class II interacting protein from the literature is the microtubule-associated binding protein Tau which was originally shown to bind another PPIase-deficient binding mutation (K63A) with the same affinity as wild-type (*35*). This would be similar to the result we obtained with the R68/69A mutation and NonO. Furthermore, the effect of Pin1 on Tau has been extensively studied in cells and the Pin1-Tau interaction has been studied *in vitro* using peptides based on the two Pin1 binding sites in Tau, T212 and T231 (which contains a proline at the +1 position) (*25, 36*).

To this end, we have already obtained N-terminally tagged GFP constructs of all six isoforms of Tau. Although studies with Pin1 and Tau are often performed in the context of healthy versus degenerated neurons, the original Pin1-Tau interaction was analyzed after phosphorylation with a mitotic lysate (*35*). This implies that experiments to examine the interactions between Pin1 and Tau could be done in the same manner as those in Chapter 4 where the Tau construct is first transfected into cells and cells are then arrested in mitosis using nocodazole. After cell lysis, Pin1-interacting proteins can then be isolated using GST pull-downs with Pin1 wild-type and mutant proteins and the interaction between the Pin1 protein and Tau protein analyzed by Western Blotting with a specific antibody. As in Chapter 4, we can further characterize the Pin1-Tau interaction by specifically mutating the Pin1 binding sites in Tau (eg. T212A, T231A or P233A) and examining their interaction with Pin1 wild-type and mutant proteins. Moreover, the interaction of Pin1 with Tau has been shown to promote its dephosphorylation by PP2A *in vitro* and enhance its ability to bind microtubules. We propose examining how wild-type or mutant forms of Pin1 affect the dephosphorylation of Tau or its mutations *in vitro* by mixing P^{32} phosphorylated Tau (generated after phosphorylation of recombinant Tau with a mitotic lysate), Pin1 and PP2A and examining the amount of phosphate in Tau

over time via autoradiography. The binding between Tau and microtubules in the presence of Pin1 could be analyzed *in vitro* (*35*) or in cells using immunoprecipitation or colocalization.

Another possible way to look at the binding mechanism of Pin1 is to investigate the interdomain linker which is different between human and fungal homologues of Pin1. By swapping the short, flexible linker of human Pin1 with the longer structured linker of *C. albicans* Ess1, we could see, by using pull-downs as in Chapter 4, if target interactions change. Alternatively, we could examine linker function in our yeast system. Previously, Gemmil *et al.*, using a galactose-inducible system, found that less than 400 molecules of wild-type Ess1 were needed for viability in *S. cerevisiae (37)*. It would be interesting to know if different linkers have different efficiencies *in vivo* by plating yeast containing various galactose-induced linker constructs on decreasing amounts of inducer.

b) Pin1 function in mammalian cells

Our test of Pin1 function was a plasmid shuffling assay set up in yeast. However, since we were using human Pin1, it would be interesting to see how our mutations function in mammalian cells. Although genetic manipulation is not as easy in mammalian cells, the use of RNAi may allow us to set up a similar system to what was used in yeast. Previous studies using RNAi have shown that knockdown of Pin1 can lead to cell cycle arrest, decreased cell growth, apoptosis, and fewer MPM-2 reactive epitopes in mitotic cells (*7, 38, 39*). Currently in the lab, we have obtained shRNA constructs which can knockdown Pin1 expression at the protein level through targeting of the 3'-untranslated region of endogenous Pin1 mRNA (E. Parker, unpublished data). As our exogenous FLAG-Pin1 constructs which were previously made in the lab do not contain the 3'-UTR, their expression in cells should not be affected by the shRNA allowing us to

re-express the Pin1 protein of our choice. Although previous work with these constructs in the lab suggest that mammalian cells may adapt to the partial knockdown of Pin1, we also have a tetracycline-responsive system which would allow us to control the knockdown of Pin1 with tetracycline in stably transfected cells. These cells could then be transfected with various Pin1 mutants and their rate of cell growth could be measured by proliferation assays. The ability of Pin1 mutations to overcome the induction of cell cycle arrest or apoptosis could be measured by FACS analysis and the intensity of MPM-2 epitopes could be compared with those of knockdown cells using Western Blotting. In these ways, we would assess the function of our mutations in mammalian cells and compare them to the function they showed in yeast.

5.6 Perspectives

The overall objective of this thesis was to further elucidate the molecular mechanisms of Pin1 action with the goal of enhancing the basis of understanding for future studies on Pin1 and determining the potential of Pin1 as a therapeutic target. Previous studies on Pin1 provided excellent data on numerous Pin1 functions and targets for study as well as good structural resources that allowed us to make conclusions on a molecular level. As a result, data from this thesis represents a merging of two well-studied areas of Pin1 research and has yielded not only perspectives on the catalytic mechanism and binding models of Pin1, but also valuable mutations to be used as tools for future studies and new insights into Pin1 as a druggable target.

Throughout our studies, we have generated various mutations along full-length Pin1 a number of which would be useful in characterizing Pin1 binding to a target, or the need for isomerization in a process on a case-by-case basis. For example, the R68/69A

mutation has been used in previous studies as a catalytic-deficient mutation (*30, 40*). Our studies show that while R68/69A does indeed have decreased isomerase activity, it also has a much lower affinity for most Pin1 targets. Consequently, this mutation should instead be viewed as tool for examining PPIase binding to full-length targets and would be especially useful as interactions with the PPIase domain alone are undetectable in pull-downs (Figure 4.1). The C113D and C113S mutation, however, do represent catalytic-deficient mutations which do not have decreased binding to targets. Furthermore, as they represent a range of isomerase activities *in vitro*, they may be useful in proving a correlation between isomerase activity and Pin1 functions *in vivo*. While our mutations to H59 and H157 also have a range of isomerase activities *in vitro*, caution should be taken when using mutations to these residues as structural differences between the mutations may change Pin1 protein levels in the system used. Overall, we feel that the characterization of the mutations used in this thesis will be a valuable resource for future studies on Pin1.

Although Pin1 inhibitors have previously been discovered or developed, their widespread use has been hindered by either their non-specificity or membrane impermeability (*41-45*). Previous studies using RNAi that have shown that downregulation of Pin1 blocks cancer cell growth and proliferation are encouraging for the development of Pin1 therapeutics (*44, 46*). Our studies have shown that Pin1 catalysis does not proceed through a covalent mechanism, so inhibitors cannot be made to target any kind of enzyme-substrate intermediate. However, our studies have suggested that, despite their sequence variability, the other PPIases may work similarly to Pin1. This is encouraging for Pin1 as a druggable target as both CyPs and FKBPs are inhibited by immunosuppressive drugs (*14, 47, 48*).

In addition, our binding studies have shown that not all Pin1 targets are bound in the same way by the WW and PPIase domains which should be taken into consideration when designing an inhibitor specifically against one domain of Pin1. Since our binding studies have not conclusively distinguished which characteristics separate Class I and II binding sites and have not determined the physiological relevance of these two sites, it is not known whether the presence of two types of binding sites will complicate therapeutic development. However, the work does suggest that the effects of therapeutics designed against one domain of Pin1 will vary depending on the target involved and whether it is a Class I or Class II binding protein.

Collectively, the studies in this thesis have provided insights into the binding and isomerization by the WW and PPIase domains of Pin1 and have expanded the knowledge of the molecular mechanisms of Pin1 action in cells. In the future, these studies may be used to help understand the roles for Pin1 in a growing number of cell signalling processes and to develop therapeutics to target misregulated Pin1 in pathogenesis.

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