PHARMACOLOGICAL INHIBITION OF PROTEIN PHOSPHATASE-5 AND INDUCTION OF THE EXTRINSIC APOPTOTIC PATHWAY IN KIDNEY CANCER

Author: Elham F. Ahanin

A Dissertation in the Department of Biochemistry and Molecular biology

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies of State University of New York, Upstate Medical University.

Approved ___________________________
(Mehdi Mollapour, PhD)

Date _________________________________
May 5, 2023
Dedication

I dedicate this work to my husband Alireza who has been a source of strength, support, patience, and motivation for me throughout this entire experience. I am truly blessed to have you as my partner in this dance called life. To my little daughter Edna, whose birth is one of the greatest joys in my life. Both of you have been my best cheerleaders.
Table of Contents

Acknowledgements ........................................................................................................... vi

List of Figures and Tables ................................................................................................... viii

Dissertation Abstract ......................................................................................................... xi

Key Abbreviations .............................................................................................................. xii

Chapter 1- Introduction ......................................................................................................... 1

1.1 Protein Phosphatase 5 (PP5) ...................................................................................... 2

1.2 PP5 structure and function ........................................................................................ 7

1.3 PP5 activation and regulation ..................................................................................... 14

1.3.1 Impact of Hsp70 and Hsp90 chaperone machinery on regulation of PP5 .......... 14

1.3.2 TPR binding proteins and release of autoinhibitory domain ......................... 17

1.3.3 PP5 post-translational modifications ................................................................ 18

1.3.4 PP5 protein expression ......................................................................................... 20

1.4 Physiological function of PP5 ..................................................................................... 21

1.5 PP5 role in cancer ........................................................................................................ 24

1.6 PP5 role in kidney cancer .......................................................................................... 27

1.7 History of PP5 inhibitors ............................................................................................ 28

1.8 The direct involvement of PP5 in apoptosis .............................................................. 33

1.9 Concluding remarks ................................................................................................... 38
Chapter 2 - Protein Phosphatase-5 (PP5) suppresses extrinsic apoptosis pathway by mediating FADD dephosphorylation in kidney cancer ........................................ 39

2.1 Abstract .......................................................................................................................... 40

2.2 Introduction ................................................................................................................... 41

2.3 Results .......................................................................................................................... 43

  2.3.1 PP5 attenuation induces extrinsic apoptosis in clear cell renal cell carcinoma. 43
  2.3.2 PP5 interacts with FADD, RIPK1, and Caspase 8. ................................................. 43
  2.3.3 PP5 controls extrinsic apoptotic pathway by dephosphorylating FADD. ............. 46
  2.3.4 FADD binds to PP5 independent of the molecular chaperone Hsp90. ............... 51
  2.3.5 PP5 associates with intact complex II. ................................................................. 54
  2.3.6 FADD death domain is essential for its binding to PP5. ...................................... 54

2.4 Discussion .................................................................................................................... 59

Chapter 3 - Design, synthesis and development of specific small molecule inhibitors of PP5 and their effect in renal cancer ......................................................... 61

3.1 Abstract ......................................................................................................................... 62

3.2 Introduction ................................................................................................................... 63

3.3 Results .......................................................................................................................... 65

  3.3.1 Characterization of PP5 specific inhibitors ......................................................... 65
  3.3.2 PP5 inhibitors specifically bind to the phosphatase-catalytic domain .......... 71
  3.3.3 Design, synthesis and characterization of a more potent PP5 inhibitor ........... 82
3.4 Discussion ................................................................................................................... 94

Chapter 4 - Discussion .................................................................................................... 96

4.1 Discussion ................................................................................................................... 97

Supplemental Information ........................................................................................... 102

Methods .......................................................................................................................... 103

References ...................................................................................................................... 131
Acknowledgements

I am very grateful to everyone who has helped me to achieve my dream of becoming a PhD. It is with immense gratitude that I acknowledge the support and help of my supervisor Professor Mehdi Mollapour for accepting me into his lab as well as his mentorship over the last four years. He has been tremendous in guiding and helping me with my project. His guidance has shaped me into a better scientist and I am extremely thankful that I joined his lab.

I would like to express my deepest appreciation for the Department of Urology and especially Dr. Gennady Bratslavsky, whose dedication, support, and leadership has been invaluable to the team, he has truly created a family atmosphere. It has meant a lot to me to have him as a role model.

I am thankful for my advisory and dissertation committee members: Drs. Patricija van Oosten-Hawle, Mira Krendel, Alaji Bah, Mark R. Woodford, Dimitra Bourboulia, and Mehdi Mollapour. Your time and advice are greatly appreciated, and especially Dr. van Oosten-Hawle for travelling to Syracuse for the occasion.

I would like to thank everyone that has supported me and contributed to making this work possible. My sincere thanks to our collaborators, Drs. John D. Chisholm, Giorgio Colombo, and Chris Prodromou as well as a special thank you to Dr. Jasmeen Oberoi for their critical contributions to my thesis. I would like to thank Dr. Adam R. Blanden who greatly helped me with all aspects of anisotropy. I also wish to acknowledge Drs. Mark R. Woodford, and Rebecca A. Sager for their role in the completion of my work. I literally could not have done it without your support and contributions to my project. You are all amazing scientists; your advice and critiques have definitely improved the quality of my research.

I would like to give a big thank you to all the current and former members of the Mollapour, Bourboulia, and Bratslavsky labs for their previous work on this project including laying the groundwork for this thesis. I would like to acknowledge my fellow lab member Dr. Sarah J. Backe for all her help and support with my projects. She has been amazing friend and colleague who fielded all my naïve questions with patience and lending her expertise to step in and troubleshoot experiments when needed. A special thank you to SarahBeth Votra for her friendship and birthday decorations. Thank you for your emotional support and encouraging me through the tough times. Many thanks go to my colleagues Kalaivani Saravananan, Kassidy Knight, Jing Li, Farzana Khan and Jennifer Heritz, who have provided friendship and support, and with whom I have shared laughter, frustration and companionship. Thanks for all the fun times and support throughout the years. I wish you all the best in your future endeavors.
I am sincerely grateful to the College of Graduate Studies and the Department of Biochemistry and Molecular Biology for their support, especially Jennifer Brennan, Cherylene Small, Shaunna Arnold, Penny McPhilmy, and Sandy Jarvis, as they have been integral in making graduate school as smooth as possible. A special thank you to Drs. Patty Kane and Xin Jie Chen for always willing to support me during my doctoral journey. I express my sincere admiration for Dean Mark Schmitt and his outstanding leadership. Your unfailing support for students has meant everything to me.

I also have deep gratitude for my family especially my husband Alireza for supporting me during the compilation of this dissertation. I am incredibly thankful for his unconditional love, encouragement and belief in me. Without you, I would not have made it here today. I would also like to thank my little baby girl Edna who is my inspiration to achieve greatness. I am so grateful to have her in my life, being a mom has made me a fighter.

Finally, I am grateful to have had the privilege of attending the prestigious SUNY Upstate Medical University. This experience has afforded me the opportunity to work with some of the best and brightest students and faculty researchers, and provided the resources to help me achieve great success. Thank you for this opportunity.
List of Figures and Tables

Chapter 1

Figure 1.1 - The process of reversible phosphorylation ......................................................3

Figure 1.2 - The phosphoprotein phosphatase family share a common catalytic domain ...5

Figure 1.3 - Structure of human PP5 TPR domain ..............................................................8

Figure 1.4 - Structure of human PP5 domains .....................................................................9

Figure 1.5 - TPR and phosphatase domains of PP5 interact to form the autoinhibited state ............................................................................................................................................10

Figure 1.6 - Catalytic mechanism of phosphoprotein phosphatase superfamily ..........12

Figure 1.7 - Conserved active site residues within the catalytic domain of PP5 ..........13

Figure 1.8 - Association of Hsp90 with the TPR domain of PP5 activates PP5 ..........16

Figure 1.9 - Post-translational modifications provide an ON and OFF switch mechanism for PP5 regulation ..............................................................................................................19

Figure 1.10 - PP5 plays a key role multiple signaling pathways ........................................22

Figure 1.11 - PP5 is involved in initiation and progression of different types of cancer .25

Figure 1.12 – Pro-survival role of PP5 in kidney cancer .....................................................29

Table 1.1 - Inhibition of PPP catalytic subunits by natural compounds .........................31

Figure 1.13 - Schematic representation of intrinsic and extrinsic apoptotic pathway ......35

Chapter 2

Figure 2.1 - Targeting PP5 activates the extrinsic apoptotic pathway............................44
Figure 2.2 - PP5 interacts with the extrinsic apoptosis proteins FADD and RIPK1.  

Figure 2.3 - PP5 is in complex with complex II of the extrinsic apoptosis pathway.  

Figure 2.4 - PP5 mediates FADD de-phosphorylation.  

Figure 2.5 - Schematic illustration of PP5-mediated downregulation of the extrinsic apoptotic pathway.  

Figure 2.6 - FADD and PP5 interaction is independent of Hsp90.  

Figure 2.7 - PP5 mediates complex II formation.  

Figure 2.8 - Schematic representation of the interaction of FADD and RIPK1.  

Figure 2.9 - PP5 binds to death domain of FADD.  

Chapter 3  

Figure 3.1 - Design pharmacologic specific inhibitor of PP5.  

Figure 3.2 - Identification of specific pharmacologic inhibitors of PP5 in ccRCC.  

Figure 3.3 - Docking site residues within the catalytic domain of PP5.  

Figure 3.4 - The predicted structure of overlapping hit, P0, bound in the PP5 catalytic domain.  

Figure 3.5 - P0 inhibits PP5 phosphatase activity.  

Figure 3.6 - The chemical structures of the identified PP5 inhibitors.  

Figure 3.7 - Development of specific inhibitors of PP5.  

Figure 3.8 - Chemical synthesis of PP5 inhibitors.  

Figure 3.9 - Small molecule inhibitors of PP5 cause apoptosis in ccRCC.
Figure 3.10 - P5 and P13 inhibit PP5 activity ............................................................... 78

Figure 3.11 - Chemical synthesis of Biotin-P5 and P13 .................................................. 79

Figure 3.12 - PP5 binds to biotinylated P5 and P13 ....................................................... 80

Figure 3.13 - Structure-based mutations revealed the mechanism of P13 binding to PP5. ............................................................................................................................................. 81

Figure 3.14 - Chemical synthesis of fluorescently labeled P13 ...................................... 83

Figure 3.15 - Synthesis of second generation of PP5 inhibitors ........................................ 85

Figure 3.16 - Inhibitory effect of P053 compound on PP5 and induction apoptosis in VHL-null ccRCC ............................................................................................................................................. 86

Figure 3.17 - Chemical synthesis of P053 inhibitors ..................................................... 88

Figure 3.18 - P053 inhibits PP5 activity ......................................................................... 89

Figure 3.19 - P053 can compete and displace PP5 from biotin-P5 and P13. ................. 90

Figure 3.20 - Specific small molecule inhibitor of PP5 induces apoptosis in VHL-null ccRCC ............................................................................................................................................. 92

Figure 3.21 - P053 selectively caused cell death in VHL-null ccRCC cells ................. 93

Chapter 4

Figure 4.1 - Schematic representation of PP5 role in regulating apoptosis in ccRCC. .... 98

Supplementary Information

Table S1 .................................................................................................................................. 125

Table S2 .................................................................................................................................. 130
Dissertation Abstract

Title: Pharmacological inhibition of Protein Phosphatase-5 and induction of the extrinsic apoptotic pathway in kidney cancer.

Author: Elham F. Ahanin
Sponsor: Professor Mehdi Mollapour

Serine/threonine protein phosphatase-5 (PP5) is involved in tumor progression and survival, making it an attractive therapeutic target. Specific inhibition of protein phosphatases has remained challenging because of their conserved catalytic sites. PP5 contains its regulatory domains within a single polypeptide chain, making it a more desirable target. Here we used an *in silico* approach to screen and develop a selective inhibitor of PP5. Compound P053 is a competitive inhibitor of PP5 that binds to its catalytic-domain and causes apoptosis in renal cancer. We further demonstrated that PP5 interacts with FADD, RIPK1 and caspase 8, components of the extrinsic apoptotic pathway complex II. Specifically, PP5 dephosphorylates and inactivates the death effector protein FADD, preserving complex II integrity and regulating extrinsic apoptosis. Our data suggests that PP5 promotes renal cancer survival by suppressing the extrinsic apoptotic pathway. Pharmacological inhibition of PP5 activates this pathway, presenting a viable therapeutic strategy for renal cancer.
### Key Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP5</td>
<td>Protein phosphatase 5</td>
</tr>
<tr>
<td>PPP</td>
<td>Phosphoprotein phosphatases</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeats</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>Cdc37</td>
<td>Cell division cycle 37</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>CK1δ</td>
<td>Casein kinase 1δ</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>ccRCC</td>
<td>Clear renal cell carcinoma</td>
</tr>
<tr>
<td>FADD</td>
<td>FAS-associated death domain</td>
</tr>
<tr>
<td>RIPK1</td>
<td>Receptor-interacting Ser/Thr protein kinase 1</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Elham Ahanin\textsuperscript{1,2,3} and Mehdi Mollapour\textsuperscript{1,2,3}

\textsuperscript{1}Department of Urology, Syracuse, NY, 13210, USA

\textsuperscript{2}Department of Biochemistry and Molecular Biology, Syracuse, NY, 13210, USA

\textsuperscript{3}Upstate Cancer Center, Syracuse, NY, 13210, USA
Protein Phosphatase 5 (PP5)

The concept of reversible protein phosphorylation was discovered by the early work of Edmond H. Fischer and Edwin G. Krebs in their “phosphorylase” research (Fischer and Krebs, 1955, Krebs and Fischer, 1955, Kresge, et al., 2011). In summary, protein kinases act as a switch and transfer a phosphate group from ATP to a protein, converting it to a phosphoprotein and then protein phosphatases remove the phosphate group, working in opposition of kinases (Figure 1.1) (Krebs and Fischer, 1955, Krebs and Fischer, 1956, Kresge, et al., 2011, Sutherland and Wosilait, 1955). Reversible phosphorylation is a highly dynamic process and maintains the normal level of phosphorylation within a cell (Ardito, et al., 2017, Blazek, et al., 2015, Brautigan and Shenolikar, 2018, Kleiman, et al., 2011, Meeusen and Janssens, 2018). The interplay of the kinase-phosphatase has an effect on most biological functions as diverse as gene expression, signal transduction, metabolism, cellular division, and changes the structure of a protein as well as, protein binding, stability, localization and activity (Brautigan and Shenolikar, 2018, Kokot and Kohn, 2022, Ubersax and Ferrell, 2007). Imbalance between kinases’ and phosphatases’ activity can cause abnormal activity of great variety of their substrates, leading to development of cancers and many other diseases such diabetes, cardiovascular diseases, and neurodegeneration (Danielsson, et al., 2005, Grundke-Iqbal, et al., 1986, Henriques, et al., 2016, Meyerovitch, et al., 1989, Nicolaou and Kranias, 2009, Oliveira, et al., 2017, Vainonen, et al., 2021, Zanivan, et al., 2013). Therefore, understanding the crucial role of protein kinases and phosphatases in cellular processes and gaining knowledge of their function and regulation is important for the development of treatments to influence dysregulation of phosphorylated and dephosphorylated proteins. The protein phosphatases are varying
Figure 1.1 - The process of reversible phosphorylation.

Schematic representation of reversible phosphorylation. Phosphorylation involved in transferring a phosphate group from ATP to a substrate by kinases and phosphatases remove the phosphate group through a hydration reaction.
depending on their amino acid substrates and can be classified into two main families: tyrosine (Tyr) phosphatases (PTPs) and serine/threonine (Ser/Thr) phosphatases (PSPs) (Chen, et al., 2017a) and it is worth noting that phosphorylation of other amino acids was also reported but compared to Ser/Thr/Tyr these are uncommon (Hardman, et al., 2019, Ramazi and Zahiri, 2021). Phosphorylation of serine and threonine residues accounts for most of the phosphorylated sites in eukaryotes (Hanks and Hunter, 1995, Khoury, et al., 2011, Manning, et al., 2002, Olsen, et al., 2006, Ramazi and Zahiri, 2021). PSPs regulate a large number of biological processes including protein synthesis, transcription and many other biological pathways (Ceulemans and Bollen, 2004, Cohen, 2001, Cohen, 2002, Cohen, 2010, Gallego and Virshup, 2005, Hinds and Sanchez, 2008, Kamenski, et al., 2004, Moorhead, et al., 2007). Within each phosphatase family, regulatory subunits and specialized domains generate substrate diversity, modulate substrate specificity, regulate enzyme activity and subcellular localization (Gallego and Virshup, 2005, Shi, 2009). The phosphoprotein phosphatases (PPPs) are one of the three major families of PSPs (Gallego and Virshup, 2005, Shi, 2009). The PPP family contains 7 subfamilies including protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B, also known as calcineurin or PP3), protein phosphatase 4 (PP4), protein phosphatase 5 (PP5), protein phosphatase 6 (PP6), and protein phosphatase 7 (PP7, also known as PPEF) (Figure 1.2) (Cohen, 2010, Gallego and Virshup, 2005, Shi, 2009). These serine/threonine protein phosphatases consist of a conserved catalytic subunit that share a common mechanism of dephosphorylation (Cohen, 2010, Shi, 2009). The catalytic subunit associates with regulatory subunits encoded by separate genes, as well as inhibitory, scaffolding and targeting proteins to form a variety of distinct holoenzymes (Brautigan, 2013, Brautigan
Figure 1.2 - The phosphoprotein phosphatase family share a common catalytic domain.

Phosphoprotein phosphatases (PPPs) consist of seven members PP1-PP7. The catalytic domains of each member are shown in the figure. Signature sequences within the conserved catalytic domain are labeled (G, glycine; D, aspartic acid; x, any amino acid; H, histidine; V, valine; R, arginine; N, asparagine; E, glutamic acid). Metal binding residues and phosphate binding residues are colored in black and pink respectively. Specific regions of each PPP family member including calcineurin-B binding (CnB); Ca\textsuperscript{2+}-calmodulin-binding motif (CaM); autoinhibitory region (AI and αJ); tetratricopeptide repeat (TPR) are highlighted. The numbers of amino acids respective to each phosphatase is given on the right.
**PP5 structure and function**

Protein Phosphatase 5 catalytic domain is structurally similar to other PPP family members, however PP5 is a unique member of PPPs because of presence of TPR motifs (residues 28–129) located in the amino-terminus domain (Das, et al., 1998). Each of TPR motif consists of a pair of antiparallel α-helices of equivalent length (~34 amino acids) and is essential for protein-protein interaction (Figure 1.3) (Das, et al., 1998, Goebl and Yanagida, 1991). In addition to the amino-terminus regulatory domain and a carboxy-terminus catalytic domain, PP5 has a flexible linker that connects these two domains plus a specific carboxy-terminus alpha J helix (αJ) subdomain (residues 490–499) (Figure 1.4) (Sager, et al., 2020, Swingle, et al., 2004, Yang, et al., 2005). Both TPR motifs and αJ helix maintain the autoinhibited form of PP5 and regulate PP5 activity (Kang, et al., 2001, Yang, et al., 2005). Removal of either the amino-terminus TPR motifs or carboxy-terminus αJ helix, stimulates the PP5 phosphatase activity (Chen and Cohen, 1997, Kang, et al., 2001, Oberoi, et al., 2016, Sinclair, et al., 1999, Yang, et al., 2005). The interaction of TPR motifs with αJ helix fold the amino-terminus domain over the catalytic domain and block access to substrates which explains the low *in vitro* basal activity of PP5 (Figure 1.5) (Kang, et al., 2001). The autoinhibition of PP5 can be relieved by the interaction between TPR motifs and other PP5 activators including the molecular chaperone heat shock protein 90 (Hsp90), heat shock protein 70 (Hsp70), and fatty acids such as arachidonic acid (Chen and Cohen, 1997, Connarn, et al., 2014, Ramsey and Chinkers, 2002, Silverstein, et al., 1997, Zeke, et al., 2005). PP5-mediated hydrolysis of phosphoprotein substrates is regulated by a conserved active site and the bi-metal center containing two metal ions (M^1 and M^2) bound to water molecules (W^1 and W^2) (Oberoi, et al., 2016, Swingle, et al., 2004). The crystal
Figure 1.3 - Structure of human PP5 TPR domain.

Ribbon representation of PP5 TPR domains (PDB: 1A17) visualized on PyMOL v4.6.0.

Each TPR domain are colored in green, cyan, and orange.
Figure 1.4 - Structure of human PP5 domains.

Structure of human PP5 (PDB: 1WAO) with the TPR and catalytic domain are colored green and blue, respectively. The carboxy terminus subdomain αJ helix is in red.
Figure 1.5 - TPR and phosphatase domains of PP5 interact to form the autoinhibited state.

Schematic representation of PP5 domains. The TPR domain interacts with the carboxy terminus alpha J helix (αJ) to maintain PP5 in an autoinhibited state. **Figure adapted from** (Sager, et al., 2020).
structure of PP5 revealed that PP5 coordinates with Mn$^{2+}$, Zn$^{2+}$, and Fe$^{2+}$ but often prefer Mn$^{2+}$ (Oberoi, et al., 2016, Swingle, et al., 2004). The two metal ions are necessary to coordinate with the substrate phosphate group for hydrolysis of phosphoprotein substrates (Swingle, et al., 2004). The interaction of metal ions with water molecules are likely involved in nucleophilic attack by a metal-ligated water molecule (Figure 1.6) (Swingle, et al., 2004). PP5 utilizes several key residues within the catalytic domain of PP5 to form an active site for direct transfer of the phosphate group of a substrate (Swingle, et al., 2004). The substrate-binding pocket is defined by 10 conserved amino acids including D242-H244-D271-D274-R275-N303-H304-H352-R400-H427 (Figure 1.7) (Sager, et al., 2020, Swingle, et al., 2004). Of these 10 active site residues, D242, H244, D271, N303, H352, and H427 have shown to be important for coordinating with M$^1$ and M$^2$ and R275, N303, R400, and H304 help positioning the phosphate ion via direct hydrogen bond interaction with all four oxygen atoms (O$^1$, O$^2$, O$^3$, and O$^4$) of the target phosphate ion respectively (Sager, et al., 2020, Swingle, et al., 2004). Each metal ion is coordinated with one oxygen atom (O$^1$-M$^1$:M$^2$-O$^2$) of the phosphate ion and stabilized by the interaction of D242 and H352 with the metal ions. D271 and W$^1$ coordinate with both metal ions, D242, H244, and W$^2$ coordinate with M$^1$, and N303, H352, and H427 coordinate M$^2$ (Sager, et al., 2020, Swingle, et al., 2004). Although D274 is the only residue among the active site motif that does not display any direct interaction with metal ions, water molecules or the phosphate ion, it forms a hydrogen bond with H304 which is both critical for PP5 function and activity (Sager, et al., 2020, Swingle, et al., 2004). The crystal structure of the catalytic domain of PP5 in complex with a peptide of its substrate cell division cycle 37 (Cdc37) provides more detail in the context of substrate binding (Oberoi, et al., 2016). In the phosphatase domain,
Figure 1.6 - Catalytic mechanism of phosphoprotein phosphatase superfamily.

A single step mechanism of catalytic reaction by PPP. Metal (M1 and M2) bound water acts as a nucleophile to attack the phosphorus atom of the substrate phosphate.
Figure 1.7 - Conserved active site residues within the catalytic domain of PP5.

View of PP5 catalytic domain (PDB:3H60) in gray with active site residues shown in stick (orange). Metal ions are shown in purple spheres.
N308, M309, Y313, W386, and Y451 are found to be involved in the catalytic mechanism. The PP5 substrate, Cdc37, coordinates directly with PP5 through N308 and Y313. M309 and W386 provide van der Waals interactions, while Y451 forms hydrogen bonds with R275 and a water molecule (Oberoi, et al., 2016). In addition, an exciting new study presented the cryo-EM structure of PP5 in complex with Hsp90-Cdc37 associated with a kinase and explored how the catalytic domain of PP5 positions within the complex to efficiently dephosphorylate substrates (Oberoi, et al., 2022). Further mutational studies indicate that metal coordinating residues H244, D271, N303, and H427 are also critical for the catalytic function of PP5 (Hamilton, et al., 2018). Mutations of N308, M309, Y313, and W386 within the catalytic pocket strongly perturbed PP5 phosphatase activity (Oberoi, et al., 2016). K97 has also been shown to alter the catalytic activity of PP5. Additionally, K32, R74, K97, and R101 located in the N-terminal TPR region of PP5 are involved in the interaction of PP5 TPR domain with Hsp90 (Russell, et al., 1999). Therefore, mutation of these key residues in the TPR region of PP5 could prevent the interaction of PP5 with its known substrates as has been shown for Cdc37 (Skarra, et al., 2011, Vaughan, et al., 2008).

**PP5 activation and regulation**

Regulation of PP5 phosphatase activity is achieved at multiple layers including post translational modifications and molecular chaperones that ultimately leads to release of autoinhibition via structural change. These aspects are discussed in detail below.

*Impact of Hsp70 and Hsp90 chaperone machinery on regulation of PP5*

As discussed above, PP5 generally has low basal activity and the primary structure of PP5 revealed that the PP5 TPR motif at its amino-terminus interacts with the αJ-helix in the
carboxy-terminus (H Kang 2001, Swingle, et al., 2004). This autoinhibitory state prevents substrates from entering the active site of PP5 (Cliff, et al., 2006, H Kang 2001, Haslbeck, et al., 2015, Ramsey and Chinkers, 2002, Yang, et al., 2005). PP5 TPR motifs act as a regulatory domain and is tightly involved in regulation of PP5 activation. In addition to the inhibitory role of the TPR domain in PP5 activation, the best characterization of the TPR domain is mediating the protein-protein interaction (Das, et al., 1998). Binding of PP5 TPR domain with the carboxy-terminus EEVD TPR-binding motif of Hsp90 and Hsp70 releases its autoinhibition and stimulates the phosphatase activity of PP5 (Figure 1.8) (Connarn, et al., 2014, Haslbeck, et al., 2015, Russell, et al., 1999, Vaughan, et al., 2008, Yang, et al., 2005, Zeke, et al., 2005). PP5 belongs to the TPR domain-containing co-chaperone family of the molecular chaperones Hsp90 and Hsp70 (Backe, et al., 2023, Sager, et al., 2019, Vaughan, et al., 2008). As a result of PP5 co-chaperone function, PP5 regulates the chaperone cycle through dephosphorylating the chaperone, co-chaperones such as Cdc37 and folliculin-interacting protein 1 (FNIP1) or the chaperone clients (Backe, et al., 2023, Sager, et al., 2019, Vaughan, et al., 2008). In this model the interaction of PP5 with Hsp90 can efficiently dephosphorylate co-chaperones Cdc37 and FNIP1 (Sager, et al., 2019, Vaughan, et al., 2008). FNIP1 dephosphorylation by PP5 disrupts Hsp90 binding and affects FNIP1 stability which leads to FNIP1 proteasome-mediated degradation (Sager, et al., 2019). In contrast, PP5-mediated dephosphorylation of Cdc37 is required for the release of Hsp90 substrates that are collectively referred to as clients (Golden, et al., 2008b, Vaughan, et al., 2008). Hsp90 clients including kinases and steroid hormone receptors, such as glucocorticoid receptor (GR), rely on chaperone machinery for proper folding, maturation and activation (Backe, et al., 2023, Silverstein, et al., 1997). The interaction of Hsp90 with
Figure 1.8 - Association of Hsp90 with the TPR domain of PP5 activates PP5.

On the left PP5 is shown in its autoinhibited state which the interaction of αJ-helix and TPR domain helps to maintain PP5 in a closed and inactive conformation. On the right interaction of carboxy terminus MEEVD motif of Hsp90 with TPR domain of PP5 release the autoinhibited state and activates PP5. **Figure adapted from (Sager, et al., 2020).**
PP5 not only releases the autoinhibition of PP5, but also mediates chaperoning of numerous Hsp90 clients (Backe, et al., 2023, Ramsey and Chinkers, 2002, Silverstein, et al., 1997, Vaughan, et al., 2008, Wandinger, et al., 2006, Yang, et al., 2005). Therefore, Hsp90 interaction with PP5 promotes PP5-mediated dephosphorylation of its substrates and provides access of PP5 to the substrates. Additionally, the structures of PP5 in the context of Hsp90-Cdc37 complex and kinase clients BRAF and CRAF revealed how PP5 operates in the complex to dephosphorylate the chaperone, co-chaperone and kinase clients (Jaime-Garza, et al., 2023, Oberoi, et al., 2022). As Hsp90 is a dimer, following binding of PP5 to either of the C-terminal MEEVD motifs of Hsp90, the phosphatase domain can be positioned differently towards the kinase clients and Cdc37 (Oberoi, et al., 2022, Prodromou and Pearl, 2003). Within the Hsp90 complex, PP5 can exist in either an open (active) or closed (autoinhibited) confirmation and switch between the two MEEVD motifs of Hsp90 (Oberoi, et al., 2022). In a closed conformation, PP5 binds to Hsp90 in a way that the phosphatase domain of PP5 is still associated with TPR motifs. While in an open conformation, PP5 binds to the other TPR-binding site of the Hsp90 dimer where the phosphatase domain can rotate towards kinase clients and Cdc37 to access the substrate phosphorylation sites (Oberoi, et al., 2022). Overall, TPR-mediated binding of PP5 to Hsp90 provides a structural platform for the phosphatase activity of PP5.

**TPR binding proteins and release of autoinhibitory domain**

In addition to molecular chaperones, PP5 interacts with a number of proteins through its TPR domain (Sager, et al., 2020). Additionally, polyunsaturated fatty acids and fatty acid-CoA esters are other cellular factors that are known to bind to the TPR domain and have
also been described to activate PP5 \textit{in vitro} (Chatterjee, et al., 2010, Chen and Cohen, 1997, Ramsey and Chinkers, 2002, Sinclair, et al., 1999, Yang, et al., 2005). S100 protein is another TPR interacting protein that has the ability to modulate PP5 activity, however this has been shown to be abolished by oxidative stress (Yamaguchi, et al., 2012). Additionally, the small GTP-binding protein Rac1 interacts with the TPR domain of PP5 and stimulates PP5 phosphatase activity by relaxing the autoinhibited state (Gentile, et al., 2006). Small molecule activators of PP5 have also been found to interfere with the TPR domain and relax the autoinhibited state of PP5 (Haslbeck, et al., 2015). Taking together, TPR-mediated protein-protein interactions can modulate PP5 activity.

\textbf{PP5 post-translational modifications}

Post-translational modifications (PTMs) are another regulatory layer involved in regulation of PP5 stability and activity (Sager, et al., 2020). Recent work has shown that PTMs of PP5 play a major role in its switching “on” and “off” in an Hsp90 independent manner in cells (Dushukyan, et al., 2017). Casein kinase 1δ (CK1δ)-mediated phosphorylation of T362 in the catalytic domain of PP5 activates the phosphatase (\textbf{Figure 1.9}) (Dushukyan, et al., 2017). Additionally, the tumor suppressor Von Hippel-Lindau (VHL) is involved in ubiquitination and subsequent proteasomal degradation of PP5 in a hypoxia- and prolyl-hydroxylation-independent manner in normal cells therefore providing an “off” switch for PP5 (\textbf{Figure 1.9}) (Dushukyan, et al., 2017). VHL targets K185 and K199 residues of PP5 for multi-monoubiquitylation \textit{in vitro} through its E3 ubiquitin ligase activity and cells lacking VHL were defective in ubiquitination of PP5 (Dushukyan, et al., 2017). Together, these PTMs appear to be crucial for regulating PP5 stability and activity.
Figure 1.9 - Post-translational modifications provide an ON and OFF switch mechanism for PP5 regulation.

The “ON” switch mechanism is through CK1δ and phosphorylation of T362 in the catalytic domain of PP5 leads to PP5 activation. On the other hand, the “OFF” switch mechanism is through VHL and ubiquitination of K185 and K199 on PP5 targets the protein for degradation in the proteasome. **Figure adapted from** (Sager, et al., 2020).
**PP5 protein expression**

There are numerous factors that affect PP5 protein expression. Hypoxia appears to be a factor involved in transcription of the gene encoding PP5, *(PPP5C)* (Golden, et al., 2004). As a result of deprivation of oxygen, the adaptive hypoxic response regulates the expression of many gene products in order to promote cell survival (Corrado and Fontana, 2020). During hypoxia, hypoxia-inducible factor 1 (HIF1) transcription factor activates and orchestrates cellular adaptation in response to alterations in cellular oxygen level (Corrado and Fontana, 2020). Upregulation of HIF1 in hypoxic conditions influences transcription of PP5 through binding to HIF1 response element within the PP5 gene promoter which causes an increase in PP5 transcription (Golden, et al., 2004). Like HIF1, p53 is another transcription factor and a key mediator of various cellular and genomic stress (Sullivan, et al., 2018). The presence of two p53 binding sites in the PP5 promoter region confirmed the role of p53 in PP5 repression (Wang, et al., 2018). Furthermore, PP5 expression was significantly upregulated in p53 deficient mice (Wang, et al., 2018). It is also noteworthy to mention the presence of regulatory interplay between PP5 and p53 in cells. PP5 mediates the inhibition of p53 function by direct dephosphorylation of p53, and PP5 deficient mice exhibit elevated p53 expression (Sager, et al., 2020, Wang, et al., 2018). In addition, PP5 promoter displays a functional estrogen response element causing estrogen-induced PP5 expression (Urban, et al., 2001). Together, PP5 expression has been shown to be influenced by many cellular factors therefore, understanding the biological actions of PP5 will provide an insight into its role in human cancers.
Physiological function of PP5

PP5 functions in many signaling pathways including hormone signaling, proliferation, apoptosis, DNA damage repair and cell cycle control (Figure 1.10) (Hinds and Sanchez, 2008, Sager, et al., 2020). PP5 associates with a number of stress-induced protein complexes such as ASK1, Raf1, ATM, ATR, DNA-PKcs, and IKKβ which indicate the diverse biological activity of PP5 (Ali, et al., 2004, Ham, et al., 2010, K Morita 2001, von Kriegsheim, et al., 2006, Wechsler, et al., 2004, Zhang, et al., 2005). It has been reported that PP5 is able to bind to apoptosis signal regulating kinase 1 (ASK1), a mammalian MAPKK kinase involved in JNK and p38 pathways (K Morita 2001). PP5 directly dephosphorylates ASK1 and thereby inactivates ASK1-dependent apoptosis in response to oxidative stress which is critical for survival of cells in hypoxia (Golden, et al., 2004, K Morita 2001). Moreover, PP5 dephosphorylate Raf-1 and negatively regulate downstream Raf-MEK-ERK pathway (von Kriegsheim, et al., 2006). Additionally, PP5 has been shown to interact with two related checkpoint kinases ATM and ATR which are known to regulate DNA damage-induced cell cycle control checkpoints (Ali, et al., 2004, Zhang, et al., 2005). Additionally, negative feedback regulation of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) by PP5 has been reported in response to DNA damage (Wechsler, et al., 2004). DNA-PKcs participate in repairing DNA double-strand breaks and PP5 phosphatase activity acts as a suppressor of DNA-PKcs activity (Goodwin and Knudsen, 2014). A further study, also uncovered the role of PP5 in DNA damage response. PP5 participates in regulation of p53-binding protein 1 (53BP1) which is recruited into DNA damage sites following DNA damage (Kang, et al., 2009). Dephosphorylation of 53BP1 via PP5 leading to be 53BP1 release from DNA damage sites after DNA repair process
Figure 1.10 - PP5 plays a key role in multiple signaling pathways.

PP5 regulates many signaling pathways as shown in the figure.
(Kang, et al., 2009). PP5 also interacts with the cell division cycle (CDC) proteins CDC16 and CDC27, which are subunits of the anaphase-promoting complex (APC) (Ollendorff and Donoghue, 1997). APC is critical during cell division and required for anaphase initiation and exit from mitosis (Barford, 2011). Recruitment of PP5 to APC complex through interaction with CDC16 and CDC27 may play a role in regulation of APC function and cell progression through the mitosis (Ollendorff and Donoghue, 1997). The hormone signaling pathways have also been reported to correlate with phosphatase activity of PP5. Interaction of PP5 with two members of steroid receptor family, estrogen receptor (ER) and glucocorticoid receptor (GR), through a Hsp90-based chaperone system regulates steroid hormone signaling and cell growth (Ikeda, et al., 2004, Schopf, et al., 2017, Wang, et al., 2007). Phosphorylated GR modulates the transcription of GR-dependent genes and maintaining the level of GR phosphorylation by PP5 regulates GR transcriptional activity (Bouazza, et al., 2012, Wang, et al., 2007, Zhang, et al., 2009). In addition, ER dephosphorylation through interaction with PP5 results in decreased ER transcriptional activity (Ikeda, et al., 2004, Sanchez, 2012). Additionally, there is considerable evidence for involvement of PP5 in nuclear factor kappa B (NF-κB) activation (Chiang, et al., 2011). In general, activation of NF-κB requires phosphorylation of the NF-κB inhibitor (IκB) by the IκB kinase (IKKβ) (Israel, 2010). Association of PP5 with G4-1, a regulatory subunit of PP2A that physically interacts with IKKβ leads to reduced phosphorylation of IκB and negatively regulates NF-κB activation (Chiang, et al., 2011). Overall, the role of PP5 in many various signaling pathways emphasize the significance of this phosphatase as a key regulatory protein in signaling cascades.
**PP5 role in cancer**

The multi-tasking role of PP5 appears to play a role in tumor initiation, progression and metastasis (Dushukyan, et al., 2017, Golden, et al., 2008a, Li, et al., 2015, Sager, et al., 2020, Wang, et al., 2018). The elevated level of PP5 has been reported to be associated with many cancers as shown in Figure 1.11 (Sager, et al., 2020). The result obtained from mantle-cell lymphoma (MCL) patients tumor samples, displayed the elevated expression of PP5 (Ghobrial, et al., 2005). In human breast adenocarcinoma (MCF-7) cell lines and in a xenograft mouse model, overexpression of PP5 strongly associated with an increase in tumor growth and proliferation (Golden, et al., 2008a, Sager, et al., 2020). The aberrant PP5 level was also correlated with metastatic disease in patients with breast cancer (Golden, et al., 2008a). Most breast cancer cells express ER that acts as a transcription factor for genes involved in survival, proliferation, and tumor growth (Allred, et al., 2004, Hua, et al., 2018, Yue, et al., 2013). The relationship between PP5 expression and cell growth was established through the ability of estrogen to induce PP5 expression and subsequently led to activation of survival pathways (Golden, et al., 2004, Urban, et al., 2001). PP5 directly associates with ER, and inhibition of PP5 expression inhibits proliferation of estrogen-dependent MCF-7 cells (Urban, et al., 2001). In contrast, long-term overexpression of PP5 may render breast cancer cells ER-independent, contributing to carcinogenesis (Urban, et al., 2001). The ER-independent, carcinogenic effects of PP5 may also be due to the regulatory mechanism between PP5 and ER (Ikeda, et al., 2004, Sanchez, 2012). Although elevated PP5 level has been linked to estrogen and the activity of ER, PP5 phosphatase activity toward ER has been shown to decrease ER transcription (Ikeda, et al., 2004). This imply a possible negative feedback loop in which ER increases...
Figure 1.11 - PP5 is involved in initiation and progression of different types of cancer.

PP5 plays a significant role in many cancers indicated in the figure.
PP5 level, whereby PP5 acts as a negative regulator of ER activity similar to regulatory interplay between PP5 and p53 tumor suppressor. PP5 deficient mice displayed higher level of p53 and had a delayed tumor onset (Wang, et al., 2018). Prostate cancer is another hormone responsive tumor and the androgen receptor (AR) contributes to the development and progression of the disease (Fujita and Nonomura, 2019). Prostate cancer cell lines showed high levels of PP5 and despite the involvement of PP5 in chaperoning of steroid hormone receptors such as GR, the link between PP5 and AR has not been yet found (Periyasamy, et al., 2007). Interestingly, deregulation of the estrogen signaling pathway drives carcinogenesis in lung cancer (Slowikowski, et al., 2017). PP5 is highly expressed in non-small cell lung cancer (NSCLC) cells and the elevated level of PP5 in non-small cell lung cancer (NSCLC) cells is possibly due to the high estrogen activity (Hsieh, et al., 2017b). Inhibition of PP5 using a pan protein phosphatase inhibitor, cantharidin, induced apoptosis through targeting PP5-AMPK axis in NSCLC cells (Hsieh, et al., 2017b). PP5 inhibition increased p-AMPK in tumor samples and in turn activation of MAPK signaling effectively suppressed tumor growth (Hsieh, et al., 2017b). Similarly, PP5 knockdown in hepatocellular carcinoma (HCC) cells significantly inhibited growth of the cells through activation of AMPK signaling (Chen, et al., 2017b). In further support, increased PP5 level in HCC was linked to a worse survival rate in HCC patients (Chen, et al., 2017b). PP5 knockdown in HCC cells significantly inhibited growth of HCC cells through activation of AMPK signaling (Chen, et al., 2017b). Additionally, palbociclib, FDA approved CDK4/6 inhibitor, significantly stimulated AMPK phosphorylation through inhibition of PP5 phosphatase activity in HCC cells (Hsieh, et al., 2017a). Palbociclib was also shown to inhibit ATM-mediated DNA damage by blocking the activity of PP5 in
cholangiocarcinoma (CCA) cells (Huang, et al., 2018). Overexpression of PP5 in associated with CCA tumorigenesis and inhibition of PP5 activity was shown to enhance AMPK phosphorylation in CCA cells and tumor xenografts (Hu, et al., 2018). Furthermore, overexpression of PP5 in HT-29 colorectal cancer (CRC) cells and subsequent dephosphorylation of DNA-PKcs, significantly increase sensitivity of these cells to Way-600, a potent mTOR inhibitor (Wu, et al., 2015). In contrast, knockdown of PP5 expression suppressed CRC cells proliferation and colony formation by inducing G0/G1 phase cell cycle arrest and apoptosis (Wang, et al., 2015). Additionally, knockdown studies have shown decreased proliferation and G0/G1 cell cycle arrest in glioma, ovarian cancer, osteosarcoma and pancreas (Grankvist, et al., 2012). The oncogenic role of PP5 is consistent with the observation of PP5 overexpression in kidney cancer (Dushukyan, et al., 2017). We have previously shown that renal cancer cell lines and patient derived tumors exhibit elevated PP5 levels (Dushukyan, et al., 2017). The positive correlation between PP5 expression and cancer development, indicate that PP5 serves as the facilitator of oncogenesis (Golden, et al., 2008a, Sager, et al., 2020). Thus, PP5 may represent a promising therapeutic target in the treatment of cancer and development of PP5 specific inhibitors could aid the medical management of human cancers.

**PP5 role in kidney cancer**

The studies presented within this dissertation focus on kidney cancer or renal cell carcinoma (RCC). RCC is estimated to account for about 81,800 new renal cancers and 14,890 deaths in the United States in 2023 (Siegel, et al., 2023). Despite improvements of therapeutic options for RCC overall survival and response rate is low with advanced RCC.
Patients mostly experience subsequent tumor progression and develop drug resistance (Chowdhury and Drake, 2020, Janzen, et al., 2003, Siegel, et al., 2023). Clear renal cell carcinoma (ccRCC) is the most aggressive type of renal cancer (Hsieh, et al., 2017c, Linehan, et al., 2019). ccRCC is often driven by mutation and inactivation of the \( VHL \) gene, which encodes the recognition subunit of an E3 ubiquitin ligase complex. VHL recognizes its substrates as part of an oxygen-dependent prolyl-hydroxylase (PHD) reaction, with HIF\( \alpha \) being its most-studied substrate (Clifford, et al., 2001, Maxwell, et al., 1999). However, we recently demonstrated that PP5 is subject to ubiquitination and degradation independent of the oxygen level by the VHL (Dushukyan, et al., 2017). Previous studies, including from our group, have demonstrated an oxygen and PHD-independent function for VHL (Bluyssen, et al., 2004, Dushukyan, et al., 2017, Hasanov, et al., 2017, Woodford, et al., 2021). Mutation and inactivation of the tumor suppressor \( VHL \) is associated with increased PP5 expression and activity in ccRCC (Dushukyan, et al., 2017). However, the role of PP5 in ccRCC is poorly explored. Our group has shown \( VHL \)-null ccRCC cells have high dependency on PP5 for their survival (Dushukyan, et al., 2017). Targeting PP5 activity through down regulation of PP5 or inhibition of PP5 phosphatase activity via CK1\( \delta \) inhibition, activates the apoptosis pathway in \( VHL \)-null ccRCC (Figure 1.12) (Dushukyan, et al., 2017). Therefore, understanding the role of PP5 in renal cancer cell survival may provide a potential therapeutic strategy in ccRCC.

**History of PP5 inhibitors**

The diverse biological roles of PP5 and its involvement in various signaling pathways makes PP5 an attractive drug target for the treatment of a variety of diseases. The earliest
Figure 1.12 – Pro-survival role of PP5 in kidney cancer.

PP5 phosphorylation by CK1δ is coupled to its activity and in normal cells VHL ubiquitinates PP5 for degradation. However, in VHL-null ccRCC, PP5 phosphatase activity is increased. Inhibition of CK1δ using the small molecule IC261 or downregulation of PP5 phosphatase activity through siRNA decreases PP5 activity and induces apoptosis in VHL-null ccRCC cell lines.
discovered PP5 inhibitors are a class of natural inhibitors but without any specificity towards PP5 over other PPP family members (Honkanen and Golden, 2002, Swingle, et al., 2007, Zhang, et al., 2013, Zhang, et al., 2021). These pan inhibitors bind to the active sites of several other PPPs with nanomolar to micromolar affinities (Table 1.1) (Honkanen and Golden, 2002, Swingle, et al., 2007, Zhang, et al., 2013, Zhang, et al., 2021). The broad substrate specificity of these natural products raise concern over toxicity (Honkanen and Golden, 2002, Swingle, et al., 2007). For a long time, challenges to study and target protein phosphatases stigmatized them as “undruggable”. The challenge in inhibitor design arises from the conserved active sites within the PPPs, the diversity and a large spectrum of substrates resulting in different signaling outcomes, and the difficulty to identify substrates of specific phosphatases (Fahs, et al., 2016, Virshup and Shenolikar, 2009). To date, FDA-approved drugs targeting a phosphatase are the immunosuppressants cyclosporin A and FK506 which inhibit PP2B (Jorgensen, et al., 2003, Schwaninger, et al., 1993). More recently, LB-100 designed for the specific inhibition of PP2A entered clinical trials and is now in both phase 1b/2 (NCT03886662) and phase 2 (NCT03027388). Recent studies have shown that LB-100 also has the ability to inhibit other phosphatases including PP5 and PP1 (D'Arcy, et al., 2019a, D'Arcy, et al., 2019b). With the knowledge of the similar catalytic activity and the conserved active sites among PPPs, it is not surprising to observe the inhibitory function of LB-100 against other PPP family members. The crystal structure of PP5 in complex with LB-100 confirmed the association of PP5 with LB-100 (D'Arcy, et al., 2019a, D'Arcy, et al., 2019b). Additionally, LB-100 mimics the activity resulting from PP5 depletion (D'Arcy, et al., 2019a, D'Arcy, et al., 2019b). These findings argue the possibility that the potential anticancer activity of LB-100 may be achieved through the
Table 1.1 - Inhibition of PPP catalytic subunits by natural compounds.

The approximate IC50 provided are in nM and represents the concentration of inhibitor that causes 50% inhibition of phosphatase activity. ND, not determined.
inhibition of both PP2A and PP5 (D'Arcy, et al., 2019a, D'Arcy, et al., 2019b). Unlike other PPP subfamilies, PP5 is a multidomain phosphatase encoded by a single gene and forms one polypeptide (Chen, et al., 2017a, Shi, 2009). Thus, the unique characteristics of PP5 display a huge potential for its specific targetability.

Despite this, there is no report of specific PP5 inhibitors, although several attempts have been made to develop PP5 inhibitors. About 30 novel inhibitors of PP5 were identified using ultra-high-throughput screen which confirmed to have no effect on PP1 although still should be tested to determine their activity against other PPPs (Swingle, et al., 2017). Additionally, protein phosphatase-recruiting chimeras (PHORCs) linking a PP5 activator to an ASK1 inhibitor exhibit a promising strategy for PP5 to specifically dephosphorylate proteins of interest. PP5 is recruited to ASK1 and enables dephosphorylation of p-ASK1 and thereby inhibiting ASK1 activation and the occurrence of tumors in gastric cancer (Zhang, et al., 2023). However, considering the requirement of two ligands to recruit and link the biological effector and the protein of interest and the challenges related to the ligands with suitable affinity may still limit the application of bifunctional molecules (Hua, et al., 2022). In contrast to these inhibitors that bind to the phosphatase domain, the compound Ro 90-7501 has been shown to inhibit PP5 in a TPR-dependent manner. The TPR-dependent PP5 inhibition could be achieved through locking PP5 in the autoinhibited conformation (Hong, et al., 2017). However, further studies are required to explore the specificity of Ro 90-7501 toward PP5 (Hong, et al., 2017). Collectively, these studies provide compelling data for the possibility in the development of compounds with high selectivity and potency against PP5.
The direct involvement of PP5 in apoptosis

Aberrant PP5 activity has been linked to a wide variety of cancers and many studies indicate pivotal role of PP5 in cancer cells survival (Sager, et al., 2020). Identification of the apoptosis signaling kinase ASK1 as a physiological substrate for PP5 demonstrates the regulatory role of PP5 in apoptosis (K Morita 2001, Kutuzov, et al., 2005, Morita, et al., 2001). Although it has been shown that PP5 downregulation or depletion increases apoptosis in cancer cells, very little is known of PP5 actions or its specific role in apoptosis (Dushukyan, et al., 2017, Hu, et al., 2018, Huang, et al., 2004, Sager, et al., 2020, Wang, et al., 2015, Zheng, et al., 2016, Zhi, et al., 2015). Apoptosis is highly regulated mechanism of cell death and loss of apoptotic controls is a well-established survival mechanism in cancers (Carneiro and El-Deiry, 2020, Danial and Hockenbery, 2018). Defective apoptosis may also have pro-oncogenic functions through initiation and development of tumors (Carneiro and El-Deiry, 2020, Wong, 2011). Apoptosis can be activated via two distinct yet interconnected pathways: the intrinsic and the extrinsic signaling pathways (Danial and Hockenbery, 2018, Obeng, 2021). The intrinsic or mitochondrial pathway is activated primarily in response to nonreceptor-mediated stimuli such as cellular stresses, viral infections, DNA damage (induced by chemicals, radiation or defects in DNA repair), hypoxia, growth factor deprivation or endoplasmic reticulum (ER) stress (Danial and Hockenbery, 2018, Elmore, 2007). These stimuli induce mitochondrial outer membrane permeabilization and release of sequestered proapoptotic factors (e.g., cytochrome c) from the intermembrane space of the mitochondria into the cytosol. Conversely, the extrinsic or death receptor pathway is activated upon the stimulation of death receptors (DRs) induced by extracellular ligands including cytokines (Danial and Hockenbery, 2018, Elmore, 2007).
Both pathways initiate apoptosis through the activation of a group of cysteine proteases named caspases (Danial and Hockenbery, 2018, Elmore, 2007, Obeng, 2021). Activation of caspases produce a proteolytic cascade leading to a cell death (Figure 1.13) (Mandal, et al., 2020). In this dissertation we largely focus on the extrinsic pathway (Chapter 2) therefore, here we review only the death receptor mediated apoptosis following formation of complex II. Death receptors are type I transmembrane proteins which belong to the tumor necrosis factor receptor (TNFR) superfamily and are characterized by their intracellular death domain (DD) and cysteine-rich extracellular domains (Ivanisenko and Lavrik, 2019, Krammer, et al., 2016, Kumar, et al., 2005, Lavrik, et al., 2005, Locksley, et al., 2001). The DRs includes six members: DR1 (TNFR1), DR2 (FAS), DR3 (APO-3), DR4 (TNF-related apoptosis-inducing ligand (TRAIL-R1), DR5 (TRAIL-R2) and DR6. The DRs oligomerize and trigger by corresponding death ligands (DLs) such as TNFα, FASL, and TRAIL (Ivanisenko and Lavrik, 2019, Krammer, et al., 2016, Kumar, et al., 2005, Lavrik, et al., 2005, Locksley, et al., 2001). Following ligand engagement and DRs stimulation, adaptor protein, for example TNFR1-associated death domain (TRADD) and FAS-associated death domain (FADD) binds to DRs via their DDs and recruit more signaling proteins at the membrane. In response to which DRs is stimulated, either the death-inducing signaling complex (DISC) is formed at FAS and TRAIL receptors or complex I at the TNFR1, DR3 and DR6 receptors (Lavrik, et al., 2005). Briefly, release of DISC or complex I promotes transition into the cytosolic form called complex II which consist of receptor-interacting Ser/Thr protein kinase 1 (RIPK1), FADD and pro-caspase 8. FADD and RIPK1 both belong to a DD superfamily and interact via DD-DD interaction (Micheau and Tschopp, 2003). FADD also contains death effector domain (DED) which is
Figure 1.13 - Schematic representation of intrinsic and extrinsic apoptotic pathway.

The mechanism of apoptosis mainly consists of two core pathways; the extrinsic pathway and the intrinsic pathway. Activation of caspase-9 suggests the involvement of mitochondria in induction of apoptosis (intrinsic) and activation of caspase-8 suggests death receptor mediated apoptosis (extrinsic). The extrinsic pathway involves the binding of ligands to cell surface death receptors. Death receptors contain extracellular domains and an intracellular cytoplasmic sequence known as the death domain. Upon binding ligand, the death receptor undergoes trimerization that results in the recruitment of adaptor proteins to form a signaling complex called complex I. Complex I formation drives oligomerization
of caspase-8 and activation through self-cleavage. Activation of caspase-8 initiate apoptosis through its downstream activation of the caspases-3 and -7. In contrast, Mitochondrion-dependent apoptosis as a result of various stress signals, triggers the release of cytochrome $c$ from mitochondria into the cytosol that binds to apoptotic protease activating factor-1 (Apaf-1) and pro-caspase 9 and forms a complex called apoptosome (Elmore, 2007, Obeng, 2021). This further leads to activation of caspase-9 and subsequent activation of effector caspases such as caspase-3 and -7. Activation of caspase 8 could also lead to cleavage of Bid into its active form tBid. Active Bid then induces conformational changes in pro-apoptotic proteins Bak and Bax for the release of cytochrome $c$ from the mitochondria.
able to recruit and bind to procaspase 8 through the interactions between their individual DED domains. Formation of complex II, causes procaspase 8 cleavage and converts it to an active form which in turn, can cleave and activate caspase 3/7 for the induction of apoptosis (**Figure 1.13**) (Ivanisenko and Lavrik, 2019, Krammer, et al., 2016, Kumar, et al., 2005, Lavrik, et al., 2005, Locksley, et al., 2001, Mandal, et al., 2020). In summary, PP5 appears to be directly involved in controlling the extrinsic apoptotic pathway. More specifically, PP5 downregulates extrinsic apoptotic pathway in kidney cancer and therefore, it is an attractive therapeutic target in renal cancer.
Concluding remarks

Protein phosphatase 5 (PP5) is a serine/threonine phosphatase and a co-chaperone of heat shock protein 90 (Hsp90) that helps regulate an array of cellular functions including stress response, proliferation, apoptosis, and DNA repair. PP5 plays a significant role in survival and propagation of multiple cancers, which makes it a promising target for cancer therapy. Though there are several naturally occurring phosphatase inhibitors, none are specific for PP5. Additionally, the detailed molecular mechanism of PP5 pro-survival role in cancer has remained elusive. This thesis will address these two overarching gaps in our knowledge. Work by our group has previously solved the X-ray crystal structure of PP5 bound to its substrate peptide Cdc37 (Oberoi, et al., 2016). This information as well as other X-ray crystal structures of PP5 was used to conduct an in silico drug screen (Chapter 3). This led to identification and development of a selective and competitive inhibitor of PP5. To our knowledge, this is the first known compound that specifically targets only the PP5 phosphatase. Chapter 2 focuses on dissecting the molecular mechanism of PP5 in cancer cell survival. We previously reported the pro-survival role of PP5 in kidney cancer (Dushukyan, et al., 2017). Data presented in Chapter 2 demonstrate PP5 interaction with FADD, RIPK1 and caspase 8, components of the extrinsic apoptotic pathway complex II (Lavrik, et al., 2005). Specifically, PP5 dephosphorylates and inactivates the death effector protein FADD, preserving complex II integrity and regulating extrinsic apoptosis. Small molecule inhibition of PP5 activates this pathway, presenting a viable therapeutic strategy for renal cancer.
Chapter 2

Protein Phosphatase-5 (PP5) suppresses extrinsic apoptosis pathway by mediating FADD dephosphorylation in kidney cancer.

Elham Ahanin$^{1,2,3}$, Rebecca A. Sager$^{1,3}$, Natela Dushukyan$^{1,2,3}$, Mehdi Mollapour$^{1,2,3}$

$^1$ Department of Urology,

$^2$ Department of Biochemistry and Molecular Biology,

$^3$ Upstate Cancer Center,
Abstract

Protein phosphatase 5 (PP5) is a serine/threonine protein phosphatase involved in the maturation and activation of numerous signaling pathways essential for cancer growth. Our previous work has shown that PP5 activity is essential for the survival of clear cell renal cell carcinoma (ccRCC). Downregulation of PP5 phosphatase activity through small interfering RNA (siRNA) or pharmacologic inhibition of the PP5 activator casein kinase 1δ (CK1δ) induces apoptosis in kidney cancer. However, the detailed mechanism and whether loss of PP5 impacts extrinsic or intrinsic apoptosis pathway remains unclear. Consistent with the requirement of PP5 phosphatase activity in downregulation of apoptosis, here we observed induction of the extrinsic apoptosis pathway following PP5 downregulation in kidney cancer. Our PP5 interactome identified known apoptotic proteins, Fas-associated death domain (FADD), receptor-interacting serine/threonine protein kinase 1 (RIPK1) and caspase 8, collectively known as complex II. In this work, we revealed that PP5 interacts with caspase 8, FADD, and RIPK1, components of the extrinsic apoptotic pathway complex II. Specifically, PP5 dephosphorylates and inactivates the death effector protein FADD at S194 residue, preserving complex II integrity and regulating extrinsic apoptosis. Taken together, our data suggests that PP5 promotes cancer cell survival by suppressing the extrinsic apoptotic pathway. Delineating the specific role of PP5 in the extrinsic apoptotic pathway will provide an opportunity for therapeutic intervention in ccRCC.
Introduction

PP5 is a unique serine/threonine protein phosphatase with a low basal activity due to the autoinhibited conformation caused by the interaction between TPR domain and αJ helix subdomain of PP5 (Kang, et al., 2001, Yang, et al., 2005). The TPR domain is involved in protein-protein interaction and it is known that PP5 activation is mediated through the interaction of the TPR domain with regulatory molecules including fatty acids, Hsp90 and Hsp70 (Connarn, et al., 2014, Kang, et al., 2001, Ramsey and Chinkers, 2002, Silverstein, et al., 1997, Yang, et al., 2005). These interactions aid dephosphorylation of PP5 substrates by making the phosphatase domain available for the substrates. Phosphorylation of T362 in the catalytic domain of PP5 by CK1δ also activates the phosphatase (Dushukyan, et al., 2017). Not surprisingly, a large number of PP5 substrates have an established role in cancer cell hallmarks including steroid hormone receptors and signaling kinases (Golden, et al., 2008b, Sager, et al., 2020). Furthermore, elevated PP5 level has been implicated in wide variety of cancers (Sager, et al., 2020). We have previously shown that VHL-deficient renal cancer cell lines and patient derived ccRCC tumors exhibit elevated PP5 levels (Dushukyan, et al., 2017). Pharmacological inhibition of CK1δ or down-regulation of PP5 induced apoptosis and reduced proliferation in VHL-null ccRCC cells, therefore suggesting a pro-survival role for PP5 in kidney cancer (Dushukyan, et al., 2017). However, the precise mechanism of PP5 involvement in apoptotic pathway remains unknown. Our PP5 interactome has identified known apoptotic proteins, Fas-associated death domain (FADD), receptor-interacting serine/threonine protein kinase 1 (RIPK1) and caspase 8. These proteins belong to the complex II of the extrinsic apoptotic pathway (Delanghe, et al., 2020, Krammer, et al., 2016, Lavrik, et al., 2005, Tourneur and Chiocchia, 2010).
Further analysis demonstrated PP5 maintain the integrity of complex II by direct interaction and dephosphorylation of S194-FADD. Downregulation or inactivation of PP5 leads to hyper-phosphorylation of S194-FADD and disassembly of complex II and ultimate cleavage of caspase 8 and activation of extrinsic apoptotic pathway. Our findings directly demonstrate the mechanism of PP5 in downregulation of the extrinsic apoptotic pathway in VHL-null ccRCC. This information can be used to design a therapeutic strategy towards activating the apoptotic pathway in kidney cancer.
Results

**PP5 attenuation induces extrinsic apoptosis in clear cell renal cell carcinoma.**

Our previous work showed that silencing PP5 in VHL-null ccRCC induced apoptosis (Dushukyan, et al., 2017). Given this, we asked whether the observed apoptosis resulted from the intrinsic or extrinsic signaling pathway. Small interfering RNA (siRNA) knockdown (KD) of PP5 in the VHL-null ccRCC lines 786-O and A498 demonstrated increased cleavage of the executioner caspases 3 and 7 and the downstream target poly-ADP ribose polymerase (PARP), hallmarks of generalized cell death (Figure 2.1A). Interestingly, increased cleavage of caspase 8 but not caspase 9 indicated that PP5 KD resulted in activation of extrinsic apoptosis (Figure 2.1A).

T362-PP5 phosphorylation by CK1δ is coupled to its activity (Dushukyan, et al., 2017). We have shown that inhibition of CK1δ using the small molecule IC261 decreased PP5 activity and induced apoptosis in VHL-null ccRCC cell lines (Dushukyan, et al., 2017). Accordingly, treatment of 786-O cells with IC261 induced extrinsic apoptosis in a dose-dependent manner (Figure 2.1B). Inhibition of caspase activity using the pan-caspase inhibitor z-VAD-fmk reversed this effect (Figure 2.1C). Our data here suggests that KD or inhibition of PP5 activates the extrinsic apoptotic pathway in VHL-null ccRCC.

**PP5 interacts with FADD, RIPK1, and Caspase 8.**

To identify PP5-interacting proteins we performed LC-MS/MS to identify the global interactome of PP5 (Unpublished data from Dr. Rebecca Segar). This was achieved by over-expression and immunoprecipitation of PP5 from HEK293 cells followed by LC-MS/MS. We identified 76 proteins that interacted with PP5 in significantly higher
Figure 2.1 - Targeting PP5 activates the extrinsic apoptotic pathway.

A) PP5 was silenced by small interfering RNA (siRNA) in VHL-null ccRCC cells 786-O and A498. Induction of apoptosis was evaluated by immunoblotting using apoptotic markers as indicated. siCtrl represents the non-targeting siRNA control. GAPDH was used as a loading control.

B) Inhibition of CK1δ by indicated amounts of IC261 for 16hrs in 786-O cells. Induction of apoptotic markers was assessed by immunoblotting. GAPDH was used as a loading control. This figure was created in collaboration with Natela Dushukyan.
C) ccRCC 786-O cells were treated in presence (+) or absence (-) of 10μM apoptotic inhibitor z-VAD-fmk for 1hr followed by the addition of indicated amounts of CK1δ inhibitor, IC261, for an additional 16hrs. Induction of apoptosis was evaluated by the immunoblotting. GAPDH was used as a loading control. **This figure was created in collaboration with Natela Dushukyan.**
abundance than the empty vector (EV). The interacting proteins were involved in biological processes including cell cycle, chaperone complex, protein transport, metabolic and proteasomal process and death inducing signaling. Notably, we found PP5 interacting with extrinsic apoptotic proteins Fas-Associated by Death Domain (FADD) and Receptor Interacting Serine/Threonine Kinase 1 (RIPK1) (Figure 2.2). These proteins, along with caspase 8 comprise complex II of the extrinsic apoptotic pathway (Figure 2.3A) (Alappat, et al., 2005, Lee, et al., 2012a, Lee, et al., 2012b, Lee, et al., 2007). We further confirmed our PP5 interactome data by immunoprecipitating (IP) PP5 from 786-O cells and observing FADD, RIPK1 and caspase 8 in the co-IP by immunoblotting (Figure 2.3B).

**PP5 controls extrinsic apoptotic pathway by dephosphorylating FADD.**

Previous work has shown that phosphorylation of S194-FADD is important for its pro-apoptotic activity (Drakos, et al., 2011, Matsumura, et al., 2009, Matsuyoshi, et al., 2006, Shimada, et al., 2004, Shimada, et al., 2002), therefore we hypothesized that PP5 targets and dephosphorylates S194-FADD to suppress apoptosis. Indeed, we found that siRNA-mediated KD of PP5 in these cells led to increased phosphorylation of S194-FADD and induction of extrinsic apoptosis (Figure 2.4A). In agreement with these findings, inhibition of PP5 activity in 786-O and A498 cells using IC261 led to an increase in S194-FADD phosphorylation (Figure 2.4B). In further support of FADD as a PP5 substrate, overexpression of PP5 in 786-O cells led to decreased S194-FADD phosphorylation (Figure 2.4C). Of note, overexpression of PP5-FLAG does not appear to impact the phosphorylation of S161-RIPK1 and S166 (Figure 2.4C). The *bona fide* PP5 substrate glucocorticoid receptor (S211-GR) has been included as a control for PP5 activity (Figure
Figure 2.2 - PP5 interacts with the extrinsic apoptosis proteins FADD and RIPK1.

Immunoprecipitation (IP) of PP5 from HEK293 cells and identification of its interacting proteins using mass spectrometry. Data generated in collaboration with Dr. Rebecca A. Sager.
Figure 2.3 - PP5 is in complex with complex II of the extrinsic apoptosis pathway.

A) Schematic representation of the extrinsic apoptotic pathway. Death receptors are activated by binding of death ligands. The leads to binding of adaptors and ultimately formation of complex II containing FADD, RIPK1, and pro-caspase 8. Upon complex II formation, caspase 8 is activated and then released from the complex leading to downstream caspase induction and apoptosis.

B) Endogenous PP5 was immunoprecipitated (IP) from 786-O cells. Co-immunoprecipitation (co-IP) of FADD, RIPK1, and caspase 8 was examined by immunoblot. IgG was used as a control. GAPDH was used as a loading control.
Figure 2.4 - PP5 mediates FADD de-phosphorylation.

A) Targeted siRNA was used to silence PP5 in VHL-null ccRCC cells 786-O. S194-FADD phosphorylation was examined by immunoblotting. Induction of apoptosis was evaluated by immunoblotting using apoptotic markers as indicated. siCtrl represents the non-targeting siRNA control. GAPDH was used as a loading control.

B) CK1δ was inhibited with indicated amounts of IC261 for 24hrs in ccRCC cells 786-O and A498. Induction of apoptotic markers shown by immunoblotting using anti-
cleaved caspase-3 antibody. Phosphorylation of S194-FADD was evaluated by western blot. GAPDH was used as a loading control.

C) WT-PP5-FLAG was transiently expressed and isolated from 786-O cells. Co-IP of FADD and GR was examined by immunoblotting. Phosphorylation level of S194-FADD, S161-RIPK1 and S166-RIPK1 were assessed by immunoblot. PP5 activity was evaluated by immunoblotting for the bona fide substrate phospho-S211-GR as a control. GAPDH was used as a loading control.

D) Lysate from WT-HAP1 and PP5-KO HAP1 cells were examined for the phosphorylation level of S194-FADD by immunoblot. GAPDH was used as a loading control.
2.4C). In agreement with these findings PP5 knockout (KO) HAP1 exhibited the elevated level of S194-FADD phosphorylation (Figure 2.4D). Taken together, our findings here indicate that PP5 can potentially regulate extrinsic apoptosis by dephosphorylating FADD (Figure 2.5).

**FADD binds to PP5 independent of the molecular chaperone Hsp90.**

Current dogma suggests that PP5 substrates are delivered to the phosphatase with the assistance of the molecular chaperone of Hsp90. In order to decipher FADD binding to PP5 in either Hsp90 dependent or independent manner, we first explored the dependency of FADD on Hsp90 to identify the importance of molecular chaperone in mediating apoptosis. We treated HEK293 with the Hsp90 inhibitor SNX-2112 (Barrott, et al., 2013). Surprisingly, we found that FADD protein level was unaffected upon Hsp90 inhibition (Figure 2.6A). We used Tsc2, Akt and phospho-S473-Akt as a positive control for Hsp90 inhibition in cells due to the fact that inhibition of Hsp90 leads to degradation of their clients in the proteasome (Xu, et al., 1999). Additionally, previous work as well as our data suggest that RIPK1 is degraded with disruption of Hsp90 inhibition (Lewis, et al., 2000). We then conducted an endogenous IP of FADD and observed that FADD does not interact with Hsp90, however, it interacts with PP5 (Figure 2.6B). These data suggest that in contrast to RIPK1, FADD is not a client of Hsp90 and the stability of FADD does not depend on molecular chaperone. Our data provide further evidence that the interaction of FADD and PP5 is independent of Hsp90.
Figure 2.5 - Schematic illustration of PP5-mediated downregulation of the extrinsic apoptotic pathway.

On the left, in the absence of PP5, following activation of the death receptors and complex II formation, caspase 8 is cleaved resulting in activation of apoptosis. On the right, when PP5 associates with complex II and dephosphorylates FADD on S194, the downstream apoptotic pathway is suppressed and leads to ccRCC cell survival.
Figure 2.6 – FADD and PP5 interaction is independent of Hsp90.

A) HEK293 cells were treated with 10μM SNX2112 (Hsp90 inhibitor) for the indicated times. FADD and RIPK1 protein stability were assessed by Western blotting. Akt, phospho-S473-Akt and Tsc2 were used as positive controls. GAPDH was used as a loading control.

B) Endogenous FADD was IP from 786-O cells. Co-IP of Hsp90 and PP5 were examined by immunoblot. GAPDH was used as a loading control.
**PP5 associates with intact complex II.**

To gain further understanding of the dynamic of PP5 interaction with complex II, we used CRISPR/Cas9 mediated-knockout (KO) of PP5, FADD and RIPK1 in HAP1 cells. These haploid cell lines are great resources for gene deletion in mammalian cells (Kotecki, et al., 1999). IP of FADD from PP5 KO cells showed FADD interaction with RIPK1 was abrogated (Figure 2.7A). IP of RIPK1 from these cells also demonstrated loss of interaction with FADD (Figure 2.7A), therefore indicating that PP5 is necessary for mediating FADD:RIPK1 complex formation. We then confirmed this model in both FADD and RIPK1 KO HAP1 cells. Our data demonstrated interaction of PP5 and FADD was unaffected in RIPK1 KO cells (Figure 2.7B). Similarly, interaction of PP5 and RIPK1 was maintained in FADD KO HAP1 cells (Figure 2.7C). In conclusion, our data provide evidence for the requirement of PP5 in the interaction between FADD and RIPK1.

**FADD death domain is essential for its binding to PP5.**

Previous work showed that FADD and RIPK1 interaction is dependent on their death domains (Figure 2.8) (Meng, et al., 2018, Vanden Berghe, et al., 2004). In order to determine whether this domain is involved in complex formation with PP5 in a cellular context we created truncated FADD (FADD-ΔDD) (Figure 2.9A) and RIPK1 (RIPK1-ΔDD) (Meng, et al., 2018, Vanden Berghe, et al., 2004) (Figure 2.9B) constructs that lack this death domain. PP5 failed only to co-IP with death domain-deleted FADD (FADD-ΔDD) construct (Figure 2.9A), demonstrating the requirement of this interaction domain for complex II assembly with PP5. Taken together, our data demonstrates PP5 functions as a scaffold for maintaining complex II by directly interacting with FADD.
Figure 2.7 - PP5 mediates complex II formation.

A) Endogenous FADD (left) and RIPK1 (right) were IP from WT-HAP1 and PP5-KO HAP1 cells. Co-IPs of RIPK1 and FADD were examined by immunoblot. GAPDH was used as a loading control.

B) Endogenous FADD (left) and PP5 (right) were IP from WT-HAP1 and RIPK1-KO HAP1 cells. Co-IPs of PP5 and FADD were examined by immunoblot. GAPDH was used as a loading control.
C) Endogenous RIPK1 (left) and PP5 (right) were IP from WT-HAP1 and *FADD-KO* HAP1 cells. Co-IPs of PP5 and RIPK1 were examined by immunoblot. GAPDH was used as a loading control.
Figure 2.8 - Schematic representation of the interaction of FADD and RIPK1.

Removing death domain (ΔDD) of either FADD or RIPK1 disrupts FADD:RIPK1 interaction.
Figure 2.9 - PP5 binds to death domain of FADD.

A) FADD-FLAG and death domain deleted FADD (FADD-FLAG-ΔDD) were transiently transfected and IP from HEK293 cells. EV was used as a control. Co-IP of RIPK1 and PP5 was examined by immunoblot.

B) RIPK1-HA and death domain deleted RIPK1 (RIPK1-HA-ΔDD) were transiently transfected and isolated from HEK293 cells. EV was used as a control. Co-IP of FADD and PP5 was examined by immunoblot.
Discussion

Many signaling and stress response pathways are tightly regulated by PP5 protein (Hinds and Sanchez, 2008, Sager, et al., 2020). PP5 upregulation is linked with a wide variety of cancers (Sager, et al., 2020). Additionally, PP5 plays a significant role in proliferation and survival of multiple cancers including kidney cancer (Dushukyan, et al., 2017, Sager, et al., 2020). Previous work published from our lab has shown that downregulation of PP5 or pharmacologic inhibition of its regulator CK1δ caused induction of apoptosis (Dushukyan, et al., 2017). Indeed, this finding suggests a pro-survival role of PP5 in renal cancer and a positive function in human tumor progression. Here, we demonstrated the direct involvement of PP5 in suppressing the extrinsic apoptotic pathway in VHL-null renal cancer. In addition, we provide evidence that down-regulation of PP5 triggers caspase-dependent apoptosis. Our global interactome of PP5 identified apoptotic proteins FADD and RIPK1 as its binding partners. We further confirmed that PP5 indeed forms a complex with FADD and RIPK1 as well as caspase 8. These extrinsic apoptotic proteins form a signaling complex called complex II (Micheau and Tschopp, 2003). Formation of complex II drives activation of caspase 8 and initiate apoptosis (Mandal, et al., 2020). Our data presented here suggest that PP5 binds to FADD and consequently other components of complex II: RIPK1 and caspase 8. The presence of active PP5 in complex II seems to maintain suppression of extrinsic apoptosis in VHL-null ccRCC and blocks the cleavage of caspase 8. Phosphorylation of S194-FADD has been shown previously to be important for its pro-apoptotic activity and suppresses tumorigenesis (Jang, et al., 2011b, Shimada, et al., 2004). Here we show that PP5 dephosphorylates S194-FADD in ccRCC, consequently maintaining the integrity of complex II. Downregulation or inhibition of PP5 leads to
hyperphosphorylation of S194-FADD, cleavage of caspase 8, and induction of the extrinsic apoptotic pathway in *VHL*-null ccRCC. Additionally, lacking PP5 in *PP5 KO* HAP1 cells caused S194-FADD hyperphosphorylation. Our findings here led us to propose a model where PP5 functions as a scaffold and facilitates FADD and RIPK1 binding via their death domains. FADD and RIPK1 associate through a conserved death domain and we provided evidence that PP5 binds and dephosphorylates S194-FADD independent of Hsp90. This is essential for complex II formation and down-regulation of the apoptotic pathway in renal cancer.
Chapter 3

Design, synthesis and development of specific small molecule inhibitors of PP5 and their effect in renal cancer

Elham Ahanin\textsuperscript{1,2,3}, Rebecca A. Sager\textsuperscript{1,3}, Diana M. Dunn\textsuperscript{1,2*}, Adam R. Blanden\textsuperscript{4}, Jasmeen Oberoi\textsuperscript{5}, Chrisostomos Prodromou\textsuperscript{6}, John D. Chisholm\textsuperscript{7}, Mehdi Mollapour\textsuperscript{1,2,3}

\textsuperscript{1} Department of Urology,
\textsuperscript{2} Department of Biochemistry and Molecular Biology,
\textsuperscript{3} Upstate Cancer Center,
\textsuperscript{4} Department of Neurology, SUNY Upstate Medical University, Syracuse, NY, 13210, USA
\textsuperscript{5} Genome Damage and Stability Centre, School of Life Sciences, University of Sussex, Brighton BN1 9RQ, UK
\textsuperscript{6} School of Life Sciences, Biochemistry and Biomedicine, University of Sussex, Falmer, Brighton BN1 9QG, UK
\textsuperscript{7} Department of Chemistry, Syracuse University, Syracuse, NY 13210, USA.

* Current affiliation: Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, NY
Abstract

Dysregulation of the serine/threonine protein phosphatase-5 (PP5) has been linked to different cancers due to its functional role in cellular proliferation, cell cycle, DNA damage response and various signaling networks including steroid hormone receptor pathways. While PP5 inhibitors have great potential to become cancer therapeutics, the existing inhibitors are currently demonstrating limited or no selectivity. Protein phosphatases are considered to be ‘undruggable,’ due to the highly conserved catalytic subunits and similarity of catalytic domains between PPPs. Therefore, whether PP5 can be specifically targeted remains elusive. Here we conducted structure-based in silico screening of a library of ~3.7 million compounds, and identified a candidate (P0) as specific potential PP5 small molecule inhibitor. Validation of this potential hit confirmed the inhibition of PP5 and induction of apoptosis in the most common subtype of kidney cancer, clear cell Renal Cell Carcinoma (ccRCC). We then used cell-based analysis for evaluating the response of P0 compound and a number of similar molecules in ccRCC cells and identified P5 and P13 compounds as a potent PP5 inhibitors. Further optimization led us to develop compound P053 that specifically inhibits PP5 phosphatase activity in a competitive manner. Compound P053 also inhibited PP5 in ccRCC cell lines lacking the tumor suppressor VHL and induced the extrinsic apoptosis. Taken together, we have designed and developed a competitive inhibitor of PP5 that caused apoptosis in renal cancer.
Introduction

The level of serine/threonine protein phosphatase-5 (PP5) has been found to be elevated in human cancers, which may aid tumor growth and cancer progression (Golden, et al., 2008b, Sager, et al., 2020). Although PP5 is not an oncogene, its upregulation has demonstrated oncogenic behavior and provided a molecular signature by which cancer cells may respond to PP5-specific inhibition. Concordantly, our previous work has shown PP5 upregulation in VHL-deficient renal cancer cell lines (Dushukyan, et al., 2017). This is the most common subtype of renal cancer. Elevated levels of PP5 were also observed in patient derived ccRCC tumors lacking the tumor suppressor VHL (Dushukyan, et al., 2017). Inhibition or down-regulation of PP5 caused apoptosis in VHL-null ccRCC cells therefore demonstrating a pro-survival function for PP5 in kidney cancer (Dushukyan, et al., 2017). These findings suggest that PP5 inhibition could provide a unique and targetable therapeutic opportunity for renal cancer. However, the development of PP5 specific inhibitors and their use in treating cancer remains a challenge. Most reported inhibitors are natural products including okadaic acid, microcystin-LR, nodularin, calyculin A and tautomycetin. These natural compounds coordinate within the catalytic pocket and lack selectivity (Swingle, et al., 2007, Zhang, et al., 2013, Zhang, et al., 2021). Due to the highly conserved character of catalytic site among PPPs, PP5 inhibitors showed limited specificity and result in targeting other PPP family members (Zhang, et al., 2013, Zhang, et al., 2021). Work from our lab and others have successfully defined the elements of substrate specificity within PP5 catalytic domain and how PP5 utilizes such elements in the mechanism of substrate binding (Oberoi, et al., 2022, Oberoi, et al., 2016). To address the challenges involved in developing a specific inhibitor of PP5, we performed a structure-
based screen using our previous published work on X-ray crystal structure of PP5 (Oberoi, et al., 2016). We have designed and developed a competitive inhibitor of PP5 that inhibits PP5 phosphatase activity and causes apoptosis in renal cancer.
Results

Characterization of PP5 specific inhibitors

Since PP5 plays a major role in survival of ccRCC, we sought to design, develop, and test specific small molecule inhibitors of this phosphatase (Sager, et al., 2020). In order to identify small molecule antagonists of PP5 an in silico docking study was initiated using our previously solved X-ray crystal structure of the PP5 active site (PDB:5HPE) (Oberoi, et al., 2016). The PP5 structure allowed us to employ a virtual screening strategy to identify new inhibitors of PP5 (Figure 3.1). Docking was performed three times with three different structures, the only differences in the X-ray structures being the nature of the metal ion bound in the active site; Mn$^{2+}$ for PDB:3H60, Zn$^{2+}$ for PDB:3H68 and Fe$^{2+}$ was substituted for the Zn$^{2+}$ ion in the PDB:3H68 structure (Bertini, et al., 2009) (Figure 3.2). The threefold docking was performed due to the promiscuity of PP5 when it comes to the metal ion in the active site, which can be Zn, Mn or Fe based. The active site residues of D271, N303, H304, M309 and W386 were chosen to define the site for docking (Figure 3.3).

Conducting a virtual screen with the Zinc library of drug like compounds (~3.7 million compounds) (Bertini, et al., 2009, Irwin, 2008, Irwin, et al., 2005) and DOCK Blaster (Irwin, et al., 2009) provided a set of 200 compounds for each crystal structure that were predicted to bind to the active site of PP5. Interestingly, only one compound (P0) was found to overlap between two of these hit-sets (Mn$^{2+}$ and Zn$^{2+}$) despite the significant similarity in the crystal structures (Figure 3.4). We first treated the ccRCC cell line 786-O with the overlapping hit compound P0 and showed a dose dependent increase in phosphorylation of the known PP5 substrates phospho-S13-Cdc37 and phospho-S211-GR (Figure 3.5). This suggested inhibition of PP5-mediated dephosphorylation of these known substrates. Based
Figure 3.1 - Design pharmacologic specific inhibitor of PP5.

*In silico* docking study used to screen a library of ~3.7 million compounds using PP5 crystal structure (PDB:3H60). Top-ranked hits were filtered and used for *in vitro* experiments.
Figure 3.2 - Identification of specific pharmacologic inhibitors of PP5 in ccRCC.

Schematic workflow of in silico screening a library of ~3.7-million compounds to identify potential PP5 inhibitors. A single overlapping hit, P0 was used to identify a set of analogs for cell-based screening. Compounds 5 (P5) and 13 (P13) were selected as candidate PP5 inhibitors.
Figure 3.3 - Docking site residues within the catalytic domain of PP5.

View of PP5 catalytic domain (PDB:3H60) with docking site residues shown in stick.
Figure 3.4 - The predicted structure of overlapping hit, P0, bound in the PP5 catalytic domain.

P0 inhibitor (yellow) of PP5 visualized on PyMOL v4.6.0.
Figure 3.5 - P0 inhibits PP5 phosphatase activity.

786-O cells were treated with indicated amounts of P0 for 24hrs. DMSO was used as a control. PP5 inhibition was evaluated by immunoblotting for the *bona fide* substrates phospho-S211-GR and phospho-S13-Cdc37. GAPDH was used as a loading control. This figure was created in collaboration with Dr. Diana M. Dunn.
on the structure of P0, a number of similar molecules were purchased and assayed (Figure 3.6, compounds P1-P13) to determine which section of the molecule contained the pharmacophore. 786-O cells were treated with 20µM compounds P0-P13 for 18 hours, and induction of apoptosis and inhibition of PP5 was assessed by immunoblotting (Figure 3.7A). Compounds P4 to P13 led to marked increases in cleaved caspase 3 (indication of apoptosis) and elevated phosphorylation of PP5 substrates phospho-S13-Cdc37 and phospho-S211-GR (indication of PP5 inhibition) (Figure 3.7A). We next examined the effect of varying doses of compounds P4 to P13 on proliferation of ccRCC cells by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay (Figure 3.7B). Compounds P5 and P13 significantly inhibited the proliferation of 786-O cells (Figure 3.7B) and were therefore selected for further evaluation. These compounds were resynthesized and their structures were confirmed by 1H NMR, 13C NMR and high-resolution mass spectrometry (Figure 3.8A-D). We further examined the effect of P5 and P13 on 786-O cells by immunoblotting and demonstrate cleavage of the apoptotic markers caspase 3 and 8 and PARP (Figure 3.9A). Taken together, we have identified two potential PP5 small molecule inhibitors that led to induction of apoptosis in ccRCC cells (Figure 3.9B).

**PP5 inhibitors specifically bind to the phosphatase-catalytic domain**

We next sought to measure the inhibition of PP5 activity *in vitro* with P5 and P13. This was done using custom synthesized phospho-S211-glucocorticoid receptor (GR) peptide as a specific substrate and measuring PP5 activity by assessing free phosphate release as a result of PP5-mediated peptide dephosphorylation. As expected, our enzyme kinetics
Figure 3.6 - The chemical structures of the identified PP5 inhibitors.

Chemical structures of 14 identified candidate PP5 inhibitors from *in silico* docking that were used in cell-based screening. *This figure was created in collaboration with Professor John D. Chisholm.*
Figure 3.7 - Development of specific inhibitors of PP5.

A) 786-O cells were treated with 20µM P0-P13 for 24hrs. DMSO was used as a control. Induction of apoptosis was assessed by immunoblotting using cleaved caspase 3. PP5 inhibition was evaluated by immunoblotting for the \textit{bona fide} substrates phospho-S211-GR and phospho-S13-Cdc37. GAPDH was used as a loading control.

B) 786-O cells were treated with the indicated amounts of selected PP5 inhibitors for 48hrs. The effect of PP5 inhibitors on cell viability was assessed by MTT assay. Errors bars represent the mean ± S.D. of three independent experiments. A Student’s t-test
was performed to assess statistical significance. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

This figure was created in collaboration with Dr. Diana M. Dunn.
Figure 3.8 - Chemical synthesis of PP5 inhibitors.

A) $^1$H NMR of P5.
B) $^{13}$C NMR of P5.
C) $^1$H NMR of P13.
D) $^{13}$C NMR of P13.

This figure was created in collaboration with Professor John D. Chisholm.
Figure 3.9 - Small molecule inhibitors of PP5 cause apoptosis in ccRCC.

A) 786-O cells were treated with 10µM P5 or P13 or DMSO vehicle control for 24hrs. Induction of apoptosis was assessed by immunoblotting. GAPDH was used as a loading control.

B) The structure of PP5 catalytic domain (gray; PDB 3H60) including Mn^{2+} ions (in purple) bound to PP5 inhibitors P5 (yellow) and P13 (cyan).
confirmed P5 and P13 to be competitive inhibitors of PP5 (Figure 3.10) with $K_i$ of P5 = 277 ± 50 nM and P13 = 234 ± 50 nM. The binding data are summarized in Figure 3.10. To obtain further evidence to support the binding of P5 and P13 compounds to PP5 in vivo, we synthesized biotinylated-P5 and biotinylated-P13 (Figure 3.11A and 3.11B). For compound P5, this was accomplished by hydrolysis of the ester on P5, amide formation with a piperazine linker, and attachment of biotin to the distal piperazine nitrogen (Figure 3.11A). In the case of compound P13, an amminopropyl linker was added to the sulfonamide nitrogen, with biotin then being attached to the far end of the linker (Figure 3.11B). We next transiently expressed PP5-FLAG in HEK293 cells and then challenged the protein lysate with different amounts of biotin-P5 and biotin-P13 (Figure 3.12). Our data shows that PP5-FLAG binds to biotinylated P5 and P13. Furthermore, at 10µM biotin-P13 bound more to PP5-FLAG compared to biotin-P5 (Figure 3.12). Due to the improved binding and activity of P13 relative to P5 we chose to continue further characterization with only P13.

We then mutated docking site residues in the active site of PP5 to obtain further insight into the mechanism of P13 binding to PP5. These included H304Q (catalytically inactive), M309C (prevents dephosphorylation of substrate phospho-Ser13-Cdc37), and W386F (hyperactivity against substrates) as well as additional mutants which help coordinate substrate binding based on our previously published work: R275A, R400A and Y451F (Figure 3.13A) (Oberoi, et al., 2016). These mutants were transiently expressed in HEK293 cells, and then the protein lysates were challenged with different amounts of biotin-P13 (Figure 3.13B). Our data showed that H304Q, M309C and W386F mutants were unable to bind to biotin-P13, confirming the P13 binding sites within the PP5 protein.
Figure 3.10 - P5 and P13 inhibit PP5 activity.

PP5 enzyme kinetic data with P5 (orange line), P13 (red line) or no inhibitor (blue line) presented as a Lineweaver–Burk plot (n = 3 independent samples).
Figure 3.11 - Chemical synthesis of Biotin-P5 and P13.

A) Synthesis of biotin conjugated P5.


This figure was created in collaboration with Professor John D. Chisholm.
Figure 3.12 - PP5 binds to biotinylated P5 and P13.

Lysates from HEK293 cells transfected with PP5-FLAG were incubated with indicated amounts of biotin-labeled P5 and P13 followed by streptavidin agarose pulldown. Co-pulldown of PP5-FLAG was detected by immunoblotting.
Figure 3.13 - Structure-based mutations revealed the mechanism of P13 binding to PP5.

A) Crystal structure of PP5 active site (gray; PDB:3H60) including Mn\(^{2+}\) ions (in purple) with predicted residues responsible (orange) for contact with P13 modeled with PyMOL software (v4.6.0).

B) WT-PP5-FLAG and predicted binding residue mutants were transiently expressed in HEK293 cells. Lysate was incubated with indicated amounts of biotinylated P13 followed by streptavidin pulldown (above). PP5 binding to biotinylated P13 was examined by immunoblot. Input expression of PP5-FLAG and mutants is below. EV was used as a control.
(Figure 3.13B). Additionally, the R400A mutant had reduced binding to biotin-P13 compared to the wild-type (WT) PP5 while R275A and Y451F had increased binding (Figure 3.13B).

We next synthesized BODIPY-labelled version of compound P13 (P13-BODIPY) in order to obtain the binding affinity of this inhibitor to PP5 in vitro (Figure 3.14A). This was accomplished by coupling the azidopropyl-P13 with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY Acid). Using fluorescence anisotropy with recombinant PP5 protein (Figure 3.14B), we determined the binding affinity of P13 compound to PP5 to be 376.2 ± 60 nM (Figure 3.14C).

**Design, synthesis and characterization of a more potent PP5 inhibitor**

We next used an *in silico* docking strategy to design series of small molecule inhibitors of PP5 based on the structure of P13 as a starting point. Examination of the docking pose of compound P13 in the active site of PP5 where the isoxazole is coordinating to the metal ions yielded some ideas about modifications that could be beneficial for binding. Neighboring the binding site was a nonpolar pocket near W386 and M309, which might accommodate a tethered nonpolar group off the xylene ring of P13. Additionally, a more polar pocket near R400 and D388 might also be accessible for an alcohol or amide tethered through the sulfonamide nitrogen of P13. Using this model as a guide, a number of sulfonamide analogs were designed and docked into the PP5 active site using Autodock Vina. This docking gave a number of potential new PP5 inhibitors that were predicted to bind to the active site of PP5 with greater affinity than the parent P13 compound. This led to synthesis of a series of compounds: P052 (16), P053 (20), P058 (22), P059 (17), P062
Figure 3.14 - Chemical synthesis of fluorescently labeled P13.

A) Synthesis and structure of BODIPY-labeled P13. This figure was created in collaboration with Professor John D. Chisholm.

B) Coomassie-stained SDS PAGE gel showing purified PP5 protein. This figure was created in collaboration with Dr. Chrisostomos Prodromou and Dr. Jasmeen Oberoi.

C) P13-BODIPY binding to PP5 measured by fluorescence anisotropy. Points are replicate means ± SEM from independent trials for display. (n=2). This figure was created in collaboration with Dr. Adam R. Blanden.
(18), P070 (19), P075 (23) and P129 (21) (Figure 3.15A). Compounds 16 and 20 were synthesized from the addition of the respective aniline to the isoxazole sulfonyl chloride 15 (Figure 3.15B). Further modification of the system was accomplished by alkylation with 3-chloropropyl p-toluenesulfonate to provide the alkyl chloride 17. The chloride 17 could then be displaced with sodium azide to access azide 18, which was then reduced to the amide and acylated to provide acetamide 19. Alcohol 21 and sulfonamide 22 were prepared by alkylation of sulfonamide 16 with the appropriate alkyl halide, while nitrile 23 was prepared by displacement of alkyl chloride 17 with sodium cyanide.

We next treated the ccRCC cell line 786-O with 10µM of the compounds shown in Figure 3.15A for 18 hours and confirmed that they inhibit PP5 as evidenced by increased phosphorylation of S211-GR, which is a known substrate of PP5 (Figure 3.16A). We next showed that only compounds P053, P058, P059 and P075 had the ability to induce apoptosis in 786-O cells (Figure 3.16B). We then treated 786-O cells with different amounts of P053, P058, P059, P075 as well as the parent compound P13 for 18 hours and measured proliferation by MTT assay (Figure 3.16C). Compound P053 significantly inhibited the proliferation of 786-O cells compared to the other compounds including P13 (Figure 3.16C and Figure 3.17). Using our in vitro PP5 phosphatase specific assay we showed that P053 also is a competitive inhibitor of PP5 with K_i of 244 ± 50 nM (Figure 3.18A and Figure 3.18B). Predicted P053 binding compared to P5 and P13 within PP5 protein structure is also demonstrated (Figure 3.19A). We next challenged the protein lysate from 786-O cells with 1µM biotin-P5 and P13 followed by competition with 1µM P053 compound (Figure 3.19B). Our data showed that 1µM P053 can compete and completely displace PP5 from biotin-P5 and P13 (Figure 3.19B). This suggests higher
Figure 3.15 - Synthesis of second generation of PP5 inhibitors.

A) Chemical structures of the second generation PP5 inhibitors derived from P13.

B) Synthesis scheme of compounds 16, 17, 18, 19 and 20.

This figure was created in collaboration with Professor John D. Chisholm.
Figure 3.16 - Inhibitory effect of P053 compound on PP5 and induction apoptosis in VHL-null ccRCC.

A) 786-O cells were treated with 10µM of the indicated second generation of PP5 inhibitors for 24hrs. DMSO was used as control. PP5 inhibition was evaluated by immunoblotting for the bona fide substrate phospho-S211-GR. SE (short exposure) and LE (long exposure) of the radiographic film. GAPDH was used as a loading control.

B) 786-O cells were again treated with 10µM of the indicated second generation of PP5 inhibitors for 24hrs. DMSO was used as control. Induction of apoptosis was evaluated
by immunoblotting using apoptotic markers cleaved PARP and cleaved caspase 8. GAPDH was used as a loading control.

C) 786-O cells were treated with indicated amounts of P13, P053, P058, P059, and P075 for 24hrs and then cell proliferation was assessed by the MTT assay. A Student’s t-test was performed to assess statistical significance \(*P < 0.05; **P < 0.01\) or non-significant (ns).
Figure 3.17 - Chemical synthesis of P053 inhibitors.

A) $^1$H NMR of P053.

B) $^{13}$C NMR of P053.

This figure was created in collaboration with Professor John D. Chisholm.
Figure 3.18 - P053 inhibits PP5 activity.

A) PP5 enzyme kinetic data with P053 (purple) or no inhibitor (blue line) presented as a Lineweaver–Burk plot (n = 3 independent samples).

B) IC50 measurements for PP5 in the presence of P5 (orange line), P13 (red line), or P053 (purple line) (n=2). Data shown as mean ± S.D.
Figure 3.19 - P053 can compete and displace PP5 from biotin-P5 and P13.

A) The structure of PP5 catalytic domain (gray; PDB 3H60) bound to PP5 inhibitors P5 (yellow), P13 (cyan), and P053 (pink).

B) Lysate from 786-O cells was collected and incubated with 1μM biotinylated P5 or P13 for 1hr and then challenged with 1μM P053 for 30min. Streptavidin-coated agarose beads were used to pull down the biotinylated compounds. Co-pulldown of endogenous PP5 and PP2A was assessed by immunoblotting.
affinity of P053 towards PP5. It is also noteworthy that biotin-P5 and P13 did not bind to PP2A suggesting their specificity towards PP5 (Figure 3.19B). Our current, as well as previously published work (Dushukyan, et al., 2017), has shown that PP5 plays a pro-survival role specifically in VHL-null ccRCC cells. We further demonstrated here that P053 significantly inhibited cell proliferation in VHL-null ccRCC cells, 786-O and A498 compared to normal epithelial renal cell HK2 as well as VHL-positive ccRCC cell lines Caki-1 and Caki-2 (Figure 3.20A and Figure 3.20B). Using propidium iodide (PI) cell of the ccRCC cells treated with different amounts of P053 compound we confirmed that P053 selectively caused cell death in VHL-null ccRCC cells in a dose dependent manner (Figure 3.21A and Figure 3.21B). Taken together, we have designed, developed and synthesized a highly specific competitive inhibitor of PP5 that caused apoptosis in VHL-null ccRCC.
Figure 3.20 - Specific small molecule inhibitor of PP5 induces apoptosis in *VHL*-null ccRCC.

A) *VHL* containing HK2, Caki-2, and Caki-1 cells and *VHL*-null cells, A498, and 786-O, were treated with the indicated amount of P053 for 24hrs, and then cell proliferation was assessed by the MTT assay. A Student’s t-test was performed to assess statistical significance *P < 0.05; **P < 0.01 or non-significant (ns).

B) *VHL* protein from HEK293, HK2, Caki-1, Caki-2, A498 and 786-O cells was assessed by immunoblotting. GAPDH was used as a loading control.
Figure 3.21 - P053 selectively caused cell death in VHL-null ccRCC cells.

A) Flow-cytometric assessment of cell death in Caki-1, Caki-2, A498, and 786-O following treatment with 30μM P053 for 24hrs (blue) or 40μM for 24hrs (orange), as determined by PI staining. Pink indicates no treatment (NT).

B) Flow-cytometric assessment of cell death in HEK293, Caki-1, Caki-2, A498, and 786-O following treatment with 30μM P053 for 24hrs (blue) or 40μM for 24hrs (orange), as determined by PI staining. Pink indicates no treatment (NT). Percentage of PI stained cells as analyzed by flow cytometry was normalized to the vehicle control for each cell line individually.
Discussion

The compelling role of PP5 in cancer cell proliferation and survival as well as its unique structure makes it an attractive therapeutic target for a wide variety of cancers (D'Arcy, et al., 2019a, Sager, et al., 2020). To date, little progress has been made to the development of specific or selective inhibitors of protein phosphatase (Zhang, et al., 2013, Zhang, et al., 2021). Currently, there are no reports of compounds that specifically inhibits PP5 function within cells (Swingle, et al., 2007, Zhang, et al., 2021). However, the detailed molecular mechanism of PP5’s pro-survival role in renal cancer promoted us to screen for and identify a small molecule inhibitor for this phosphatase (Dushukyan, et al., 2017). We took advantage of available X-ray crystal structures of the PP5 active site and performed threefold docking due to the promiscuity of PP5 to the metal ion in the active site, which can be Zn, Mn or Fe based (Oberoi, et al., 2016). To define the “active site” for docking, we chose conserved active-site residues D271, N303 and H304, as these residues contact with metal ions and help position the phosphate ion as well as substrate-binding residues M309, and W386 that are also near the metal ions in the active site of PP5 and are involved in substrate recognition (Oberoi, et al., 2016, Swingle, et al., 2004). Our in silico screen and further optimization led us to the identification of 14 potential compounds. All compounds exhibited substantial effects on PP5 phosphatase activity. The treatment of 20μM compounds significantly increased intracellular phospho-S13-Cdc37 and phospho-S211-GR level bona fide PP5 substrates. Notably, P5 and P13 showed the best potency among these candidate compounds and decreased the cell viability in 786-O cells. Subsequent evidence showed that P13 binds more to PP5 after treatment of HEK293 cells with biotin P5 and P13. We also obtained similar data in treatment of 786-O cells with P5.
and P13. As shown in Figure 3.9A, P13 was significantly induced the induction of apoptotic markers compared to P5, indicating the strong antiproliferative activity of P13. In addition, P13 has high affinity (nM range) towards PP5 and appears to be in contact with the residues H304, M309, W386 and R400 within the substrate-binding pocket. These residues are highly conserved among PPP superfamily with the exception of M309 (Oberoi, et al., 2016, Swingle, et al., 2004). Moreover, the PP5 active-site residues are conserved in other phosphatases such as PP2A and interestingly, we did not observe any binding of our compounds to PP2A (Swingle, et al., 2004). Therefore, it is possible that the M309 could provide selectivity for PP5 inhibitors to distinguish the broad spectrum of the PPP superfamily. We further designed series of compounds based on the structure of P13 compound. We identified P053 to exhibit a potent activity towards inhibiting VHL-null ccRCC proliferation. Additionally, P053 competed with biotin-P5 and P13 for binding to PP5 protein, suggesting the higher affinity for the active site of PP5. Therefore, all evidence indicated P053 as a specific competitive inhibitor of PP5 with an anticancer effect in VHL-null ccRCC.

Important next steps for further evaluation of these PP5 inhibitors are to examine their pharmacokinetic and pharmacodynamic parameters as well as their effect on VHL-null ccRCC xenograft models. The impact of these inhibitors in other cancers in which PP5 has been seen to play a pro-tumorigenic role is also warranted.
Chapter 4

Discussion

Elham Ahanin$^{1,2,3}$

$^1$Department of Urology, Syracuse, NY, 13210, USA

$^2$Department of Biochemistry and Molecular Biology, Syracuse, NY, 13210, USA

$^3$Upstate Cancer Center, Syracuse, NY, 13210, USA
Discussion

The serine/threonine protein phosphatase 5 is responsible for controlling many different signaling pathways (Sager, et al., 2020). Elevated levels of PP5 in a number of cancers as well as its role in clear cell renal cell carcinoma survival makes PP5 an attractive drug target (Dushukyan, et al., 2017, Sager, et al., 2020). In this thesis we showed the induction of the extrinsic apoptosis pathway following PP5 inhibition (Figure 4.1). Although we have not identified which tumor necrosis factor including DRs and their ligands play a role in the induction of apoptosis, it has been shown that TNF-α-dependent apoptosis is inhibited in VHL-null ccRCC (Tang, et al., 2016). Similarly, TRAIL-mediated apoptosis has also been shown to be inhibited by upregulation of FADD-like apoptosis regulator (cFLIP) and HIF-2α in RCC (Isono, et al., 2018). Using sorafenib (kinase inhibitor) and bortezomib (proteasome inhibitor) which both have clinical activity in RCC sensitized renal cells to TRAIL-mediated apoptosis (Brooks, et al., 2010, Gillissen, et al., 2017). In this chapter we will discuss the possible mechanism of PP5 action in inhibiting the extrinsic apoptotic pathway. Our PP5 interactome data identified FADD, RIPK1 and caspase 8, which form complex II of the extrinsic apoptosis pathway (Alappat, et al., 2005, Lee, et al., 2012a, Lee, et al., 2012b, Lee, et al., 2007). This finding is in an agreement with the induction of the extrinsic apoptosis pathway upon PP5 inhibition. In our effort to define how PP5 is influencing the function of these apoptotic proteins in the extrinsic pathway, we established FADD as a new substrate of PP5. FADD as an adaptor molecule is well known for its contribution in death receptor-induced apoptosis and contains a DED domain that binds to caspase 8 and c-FLIP and a DD involve in binding to other DD containing proteins including DRs and RIPK1 (Carrington, et al., 2006, Delanghe, et al., 2020, Lee, et al.,
In renal cancer cells PP5 interacts with complex II of extrinsic apoptotic pathway and dephosphorylate S194-FADD which then downregulates extrinsic apoptotic pathway leading to survival. However, upon PP5 inhibition, FADD can no longer gets dephosphorylated causing induction of the extrinsic apoptosis pathway. PP5 appears to be important for preserving complex II integrity and regulating the extrinsic apoptosis pathway through facilitating the interaction of FADD and RIPK1.
Previous studies report a positive correlation between phosphorylation status and tumor suppressor activity of the FADD (Drakos, et al., 2011, Jang, et al., 2011a, Matsumura, et al., 2009, Matsuyoshi, et al., 2006, Shimada, et al., 2004, Shimada, et al., 2002). Here, we demonstrated PP5 mediated dephosphorylation and regulation of FADD. The contribution of S194-FADD phosphorylation in JNK activation and apoptosis may negatively regulate PP5 activity. This may be similar to the impact of PP5 in ASK1-dependent apoptosis (Shimada, et al., 2002). PP5 dephosphorylates both FADD and ASK1 and it is likely this leads to inhibition of cell death signaling and tumor cell survival. While FADD phosphorylation has a positive effect on apoptosis, it is possible that FADD phosphorylation status may dictate its role in ccRCC survival. It has also been shown that many ccRCCs exhibit lower levels of FADD than adjacent normal kidney, however, it is not yet clear whether this may be related to PP5 upregulation (Xu, et al., 2009). Together, the above studies demonstrate that PP5 controls extrinsic apoptotic pathway by dephosphorylating FADD. We also expand on the dynamic of PP5 interaction with complex II. Here we show PP5 knock out HAP1 cells abrogate the interaction between FADD and RIPK1, which is essential for formation of complex II and induction of apoptosis. Together, our data demonstrate that PP5 facilitates the interaction between RIPK1 and FADD and the most likely model based on our data is identification of PP5 as a “scaffold” to maintain the integrity of complex II, while simultaneously promoting downregulation of the extrinsic apoptosis pathway. PP5 achieves this by selectively dephosphorylating FADD at S194 residue, causing inhibition of pro-apoptotic activity of FADD and downregulation of apoptosis (Figure 4.1). Finally, we explore the physiological relevance of this process in kidney cancer through development of small molecule specific inhibitors of PP5. There
are several naturally occurring non-specific PP5 inhibitors (Golden, et al., 2008b, Swingle, et al., 2007, Xu, et al., 2009). These include but are not limited to: okadaic acid, microcystins, nodularin, calyculin A, tautomycin and cantharidin (Golden, et al., 2008b, Swingle, et al., 2007, Xu, et al., 2009). Due to the lack of specificity these inhibitors are not suitable as therapeutic approaches and designing specific phosphatase inhibitors is difficult due to similarities within the catalytic domains of phosphatases (McConnell and Wadzinski, 2009). The antitumor drug LB-100, which has completed a phase I clinical trial and is actively recruiting in phase 1b/2 (NCT03886662) and phase II (NCT03027388) trials, was designed as a specific PPP2AC inhibitor (clinicaltrials.gov). However, this drug also inhibits PP5 and some of the antitumor action may be ascribed to this role (D’Arcy, et al., 2019b). This highlights the difficulty in designing specific phosphatase inhibitors. However, there are several unique features within PP5 structure and function that makes it a likely druggable target (Oberoi, et al., 2022, Oberoi, et al., 2016). Therefore, we explored these structural features of PP5 and conducted an in silico screen of a library of ~3.7-million compounds to identify and design small molecules that potentially target PP5. These inhibitors have also been used as chemical tools in our study to confirmed the pro-survival role of PP5 in kidney cancer. We then applied biochemical, biophysical, and cell-based assays to design and synthesize a potent competitive inhibitor of PP5 (P053). This compound had an ability to induce apoptosis in VHL-null ccRCC cells, therefore making it an ideal candidate compound for PP5 inhibition in renal cancer. The next logical step in development of our PP5 inhibitor, is to obtain maximum tolerated dose, pharmacodynamic and pharmacokinetic properties, as well as, its impact in ccRCC xenograft model. Our
finding presented here has provided us with a better understanding of this key phosphatase in renal cancer survival and how to effectively target it.
Supplemental Information
Methods

Cell lines
Cultured human embryonic kidney (HEK293) and human kidney 2 (HK2) cells were grown in Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich), 786-O cells in Roswell Park Memorial Institute (RPMI)1640 Medium (Sigma-Aldrich), A498 cells in Minimum Essential Medium (MEM, Sigma-Aldrich), Caki-1 and Caki-2 cells in McCoy’s 5A Medium (Sigma-Aldrich) and wild-type (WT) HAP1 and knock-out (KO) cells in Isocove’s Modified Dulbecco’s Medium (IMDM, Gibco), all supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich). WT-HAP1 and KO cell lines were acquired from Horizon Discovery. All other cell lines were obtained from (American Type Culture Collection, ATCC). Cells were maintained in a CellQ incubator (Panasonic Healthcare) at 37°C in an atmosphere containing 5% CO₂.

Plasmids
For mammalian expression, pcDNA3-PP5-FLAG and the M309C and W386F point mutations were created previously (Dushukyan, et al., 2017, Oberoi, et al., 2016). Site-directed mutagenesis was performed to mutate R275A, H304Q, R400A, and Y451F residues (see Table S1) and confirmed by DNA sequencing. The pcDNA3-FLAG-FADD and pcDNA3-HA-RIPK1 were purchased from Addgene. FLAG-FADD-ΔDD as well as HA-RIPK1-ΔDD constructs were subcloned using the primers listed in Table S1. For bacterial expression, we used our previously reported human PP5 gene in pGEX6P1 plasmid with an N-terminal GST tag and C-terminal His₆ tag (Oberoi, et al., 2016).
Cell Transfection and Treatment

Cultured cells were split and then transfected the following day when about 40% confluent with each construct using Mirus TransIT-2020 (MirusBio) according to manufacturer’s protocol. Cells were incubated at 37°C and then extracted or collected for analysis after 24hrs (HEK293) or 72hrs (ccRCC cell lines). Short interfering RNA (siRNA) scramble control and PPP5C (PP5) targeting duplexes were purchased from OriGene (SKU: SR321403A, SR321403B, and SR321403C). Indicated cells were transiently transfected with the siRNA using Mirus TransIT-2020. For PP5 knock-down, either 30nM of control siRNA or 10nM of each PP5 siRNA duplex (A, B and C) were mixed prior to transfection. Cells were incubated at 37°C for 72hrs, then harvested for protein extraction. To inhibit PP5 activity, 786-O cells were incubated with indicated amount of IC261 (Abcam) for 16hrs. Blockage of caspase activity was performed by treatment with 10μM of z-VAD-fmk (Enzo Life Sciences) for 1hr followed by IC261 treatment with the indicated amount for 16hrs. Cells were then harvested for protein extraction.

Protein Extraction, Immunoprecipitation, and Immunoblotting

Protein extraction from mammalian cells was carried out using methods previously described (Mollapour, et al., 2010, Woodford, et al., 2016). Cell lysates were quantified using 1X Bradford reagent (Biorad). For immunoprecipitation, cell lysates were incubated with anti-FLAG antibody conjugated beads (Sigma) or anti-HA conjugated beads (ThermoFisher Scientific) at 4°C for 2hrs. Endogenous IPs were achieved by incubating lysate with anti-PP5 antibody (Cell Signaling), anti-FADD antibody (Cell Signaling), or anti-RIPK1 antibody (Cell Signaling) overnight followed by protein G agarose (Invitrogen)
at 4°C for 2hrs. Immunopellets were washed 4 times with fresh lysis buffer (20mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM MgCl₂, 0.1% NP40, protease inhibitor cocktail (Roche), and PhosSTOP (Roche)) and eluted in 5x Laemmli buffer. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Co-immunoprecipitated proteins or proteins from cell lysate were detected with antibodies recognizing FLAG, 6x-His (ThermoFisher Scientific), GAPDH (ENZO Life Sciences), Cdc37 (StressMarq), GR, phospho-GR (S211), PP5, caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, cleaved caspase-7, cleaved-PARP, phospho-RIPK1 (S166), phospho-FADD (S194), PP2A-C, VHL (Cell Signaling), phospho-RIPK1 (S161) (Invitrogen), PP5 and phospho-Cdc37 (S13) (Abcam). Secondary antibodies raised against mouse and rabbit (Cell Signaling) were used (See Key Resources Table, Table S1).

**Bacterial Expression and Protein Purification of PP5**

Human PPP5c was cloned into pGEX6P1 with an N-terminal GST tag and C-terminal His₆ tag. Transformed cells were grown at 37°C in LB with 100 mg/L ampicillin until OD₆₀₀ = 0.6 and induced with 1mM IPTG. Cells were harvested by centrifugation and lysed by sonication in lysis buffer (50 mM Hepes (pH 8.0), 150 mM NaCl, 0.5mM TCEP and EDTA-free protease inhibitor cocktail tablet (Roche)). Lysate was incubated with talon resin (Takara Bio) for 1hr at 4°C. The resin was washed three times with lysis buffer and PP5 was eluted with lysis buffer containing 250mM imidazole. Precision protease was added to the elution overnight to cleave the GST tag and the sample was then mixed with Glutathione Sepharose resin (Cytiva) to remove the free GST and un-cleaved protein. The
sample was applied to a Superdex S75 16/60 size exclusion column (GE Healthcare) and eluted in 100mM NaCl, 20mM Hepes pH 8, 02mM TCEP.

**Cell Viability Assay**

Renal cancer cell lines 786-O, Caki-2, Caki-1, and A498 as well as the normal renal cell line HK-2 were plated at 10,000 cells per well in 96-well plates. Cells were treated with different amounts of inhibitors (P075, P059, P058, P13, P053) and DMSO was used as control (0 μM). After 24 or 48hrs, cell viability assay was performed using the Quick Cell Proliferation Kit Plus (BioVision) according to the manufacturer’s protocol. The absorbance at 450nm was measured on a Tecan Infinite M200 Pro and proliferation rate was calculated.

**In silico Docking**

Virtual high throughput docking simulations were carried out using Dockblaster (Irwin, et al., 2009) with the Zinc library of drug like compounds (~3.7 million compounds) (Irwin, 2008, Irwin, et al., 2005). PDB structures of PP5 (PDB:3H60 and 3H66) were used as receptor structures. In preparation for docking the water molecules were eliminated, and any missing hydrogens and charges were added to the system to generate the receptor input file. The active site residues of D271, N303, H304, M309 and W386 were chosen to define the active site for docking, as these residues are nearby the active site. Once biological activity was of an inhibitor was confirmed, docking was performed again using Autodock Vina (Trott and Olson, 2010) to generate docking poses which were used to guide synthetic efforts. The Autodock docking calculation was carried out using a grid per map with 40 ×
40 × 40 Å points of (PDB: 3H60) in addition to a grid-point spacing of 0.375 Å, which was centered on the metals in the active site.

**Synthesis of Small Molecules**

*General experimental information for the synthesis of small molecules*

All anhydrous reactions were run under a positive pressure of argon. Dichloromethane (DCM) was dried by passage through an alumina column. 1,2-Dichloroethane (DCE) was freshly distilled from calcium hydride before use. Tetrahydrofuran (THF) was freshly distilled from Na/benzophenone still before use. DMF was distilled from calcium hydride under reduced pressure. Ethyl acetate (EA) and hexanes were purchased from commercial sources and used as received. Silica gel column chromatography was performed using 60 Å silica gel (230–400 mesh). Melting points were obtained on crystalline compounds and are uncorrected. The BODIPY acid 13 was prepared as reported previously (Krajcovicova, et al., 2018).

Methyl 3-aminobenzoate 2 (4.23g, 28.1 mmol) was dissolved in 52mL DMF and cooled to 0°C. Pyridine (4.1mL, 51 mmol) was then added and the mixture stirred for 10 minutes. 3,5-Dimethyl-isoxazole-4-sulfonyl chloride 1 (5.00 g, 25.5 mmol) was then added in portions over one hour. The reaction was then allowed to warm to room temperature (RT) and stirred for 16 hrs. The reaction was quenched by adding 1M HCl until the pH remained below 2 (~60mL). The reaction mixture was then taken up in EA (400mL) and washed with 1M HCl (2 x 200mL) and brine (2 x 200mL). The organic layer was dried (MgSO₄)
and concentrated to give a yellow solid. Purification using silica gel chromatography (50% EA/50% hexanes) provided pure 3 (5.65 g, 72%) as a yellow solid.

**Methyl m-(3,5-dimethyl-4-isoxazolylsulfonlamino)benzoate 3 (P5).** mp = 140-142 °C; TLC Rf = 0.45 (50% EA/50% hexanes); IR (ATR) 3226, 2970, 2359, 2341, 1692, 1588, 1339, 1306 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.84-7.81 (m, 2H), 7.79 (bs, 1H), 7.43-7.36 (m, 2H), 3.93 (s, 3H), 2.52 (s, 3H), 2.30 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 174.4, 166.6, 157.6, 136.5, 131.5, 129.9, 126.8, 125.7, 122.3, 115.4, 52.8, 12.7, 10.8; HRMS (ESI⁺) m/z calculated (calcd) for C₁₃H₁₄N₂O₅S [M + Na]⁺: 333.0516, found: 333.0515.

Potassium hydroxide (8.5g, 152 mmol) was dissolved in 40 mL of water and MeOH (160mL) was added. The ester 3 (P5) (5.2g, 16.9 mmol) was then added and the reaction mixture was stirred for 16 hrs at RT. The reaction mixture was then quenched by slowly adding 1M HCl until the pH remained below 2 (~180mL). The methanol was then removed *in vacuo* and the residue was taken up in water (200mL). This mixture was extracted with EA (3 x 150mL), and the combined organic extracts were dried (MgSO₄) and concentrated to provide carboxylic acid 4 (4.82g, 96%) as an off white solid which was used without further purification.

**m-(3,5-Dimethyl-4-isoxazolylsulfonlamino)benzoic acid 4.** mp = 184-187°C; TLC Rf = 0.51 (100% EA); IR (ATR) 3157, 2980, 2359, 1694, 1590, 1408, 1118 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 7.80-7.78 (m, 2H), 7.42-7.38 (m, 1H), 7.33-7.30 (m, 1H), 2.49 (s,
3H), 2.26 (s, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 175.3, 168.8, 158.9, 138.6, 133.2, 130.6, 127.4, 126.7, 123.1, 116.8, 12.5, 10.7.

Carboxylic acid 4 (1.0g, 3.37 mmol) was dissolved in 25mL DMF and EDCI (0.79g, 5.05 mmol), HOBT (80%, 0.95g, 6.31 mmol) and diisopropylethylamine (0.88mL, 5.05 mmol) were added. After aging for 30 min, tert-butyl piperazine-1-carboxylate (0.94g, 5.05 mmol) was then added in one portion. The reaction mixture was stirred at RT for 3 hrs, and then quenched by the addition of brine (30mL). The mixture was then taken up in EA (100 mL) and washed with water (2 x 60mL) and brine (2 x 60mL). The organic layer was then dried (MgSO$_4$) and concentrated. Purification of the residue using silica gel chromatography (60% EA/40% hexanes) gave amide 5 as a white solid (1.62 g, 94%).

**tert-Butyl 4-[m-(3,5-dimethyl-4-isoxazolylsulfonylamino)benzoyl]-1-piperazine carboxylate 5.** mp = 187-189°C; TLC Rf = 0.61 (80% EA/20% hexanes); IR (ATR) 3080, 2974, 2359, 2341, 1618, 1614, 1165, 1120 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.59 (bs, 1H), 7.32-7.30 (m, 2H), 7.15-7.12 (m, 2H), 3.76-3.37 (m, 8H), 2.49 (s, 3H), 2.28 (s, 3H), 1.47 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 173.7, 169.7, 157.6, 154.5, 137.1, 136.2, 129.5, 123.8, 123.3, 121.4, 115.6, 80.6, 47.7, 47.6, 43.5, 42.5, 28.4, 12.6, 10.8.

The Boc protected amine 5 (0.95 g, 20.43 mmol) was dissolved in 1:1 DCM:TFA (60mL) and stirred for 1 hr. The solvent was then evaporated, and the crude TFA salt was used in
the next step without further purification. Biotin (1.01g, 2.19 mmol), EDCI (0.51g, 3.3 mmol), HOBt (0.62g, 4.1 mmol) were dissolved in 20mL of DMF and diisopropylethylamine (0.77mL, 4.4 mmol) was then added. After stirring for 10 min the crude TFA salt (0.80g) was then added to the reaction mixture in portions. The reaction was then stirred for 16 hrs at RT. The reaction mixture was then taken up in brine (30mL) and extracted with EA (3 x 30mL). The combined organic layers were washed with water (2 x 30mL) and brine (2 x 30mL), dried (MgSO₄) and concentrated. Purification using silica gel chromatography (5% MeOH/95% DCM) gave the biotin-P5 6 as a white solid (0.53g, 40%).

**N-[3-(4-(4-[(3aS,4S,6aR)-2-Oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl]butanoyl)piperazine-1-carbonyl)phenyl]-3,5-dimethyl-1,2-oxazole-4-sulfonamide 6 (biotin-P5)**

TLC Rf = 0.40 (30% EA/70% toluene); IR (ATR) 3083, 2978, 2359, 2341, 1686, 1614, 1406, 1120 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.43 (t, J = 7.9 Hz, 1H), 7.27–7.24 (m, 2H), 7.19-7.18 (m, 1H), 4.49 (dd, J = 7.8, 4.7 Hz, 1H), 4.31 (dd, J = 7.8, 4.4 Hz, 1H), 3.67-3.37 (m, 8H), 3.24-3.20 (m, 3H), 2.93 (dd, J = 12.7, 4.9 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.48-2.47 (m, 5H), 2.25 (s, 3H), 1.78-1.57 (m, 4H), 1.51-1.46 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 175.2, 174.2, 171.5, 166.0, 158.9, 138.7, 137.7, 131.0, 125.0, 124.5, 116.9, 63.3, 61.6, 57.0, 46.4, 46.3, 42.8, 42.6, 41.0, 33.6, 29.8, 29.5, 26.2, 12.6, 10.8; HRMS (ESI+) m/z calcd for C₂₆H₃₄N₆O₆S₂ [M + Na]⁺: 613.1879, found: 613.1872.
3,4-Dimethylaniline 7 (1.36g, 11.2 mmol) was dissolved in 20mL DMF and cooled to 0°C. Pyridine (1.7mL, 20.4 mmol) was then added and the mixture stirred for 10 min. 3,5-Dimethyl-isoxazole-4-sulfonyl chloride 1 (2.00g, 10.2 mmol) was then added in portions over one hour. The reaction was then allowed to warm to RT and stirred for 16 hrs. The reaction was quenched by adding 1M HCl until the pH remained below 2 (~25mL). The reaction mixture was then taken up in EA (80mL) and washed with 1M HCl (2 x 50mL) and brine (2 x 50mL). The organic layer was then dried with MgSO₄. Filtration and evaporation of solvent yielded a red-brown solid which was further purified using silica gel chromatography (40% EA/60% hexanes) to provide sulfonamide 8 as a beige solid (2.04g, 73%).

(3,5-Dimethyl-4-isoxazolylsulfonyl)(3,4-xylyl)amine 8 (P13). mp = 90-92°C; TLC Rf = 0.48 (50% EA/50% hexanes); IR (ATR) 3250, 2920, 1591, 1327, 1118 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.06-7.01 (m, 2H), 6.87-6.86 (m, 1H), 6.81 (dd, J = 8.0, 2.4 Hz, 1H), 2.42 (s, 3H), 2.27 (s, 3H), 2.20 (s, 3H), 2.19 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 174, 157.8, 138.2, 135.3, 133, 130.6, 124.4, 120.5, 115.5, 19.8, 19.3, 12.6, 10.9. HRMS (ESI+) m/z calcd for C₁₃H₁₆N₂O₃S [M + H]⁺: 281.0954, found: 281.0955.

The sulfonamide 8 (P13) (1.80g, 6.42 mmol) and K₂CO₃ (1.95g, 14.12 mmol) were suspended in 22mL DMF and stirred for 15 min at RT. 3-Chloropropyl p-toluenesulfonate (2.39g, 9.63 mmol) was then added and the reaction was heated to 80°C (oil bath temperature). After 20 hrs, the reaction mixture was allowed to cool to RT and 30mL water
was added. The reaction mixture was then extracted with EA (3 x 30mL). The combined organic layers were washed with brine (100mL), dried with MgSO\textsubscript{4} and filtered. After evaporating the solvent, the residue was purified using silica gel chromatography (5% EA/95% hexanes) to provide alkyl chloride \textit{9} as a pale yellow solid (1.89g, 83%).

\textbf{(3-Chloropropyl)(3,5-dimethyl-4-isoxazolylsulfonyl)(3,4-xylyl)amine 9.} mp = 90-93°C; TLC Rf = 0.68 (30% EA/70% hexanes); IR (ATR) 2923, 2358, 1587, 1346, 1121, 689 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.09 (d, \(J = 8.0\) Hz, 1H), 6.94 (d, \(J = 2.0\) Hz, 1H), 6.85 (dd, \(J = 8.4, 2.4\) Hz, 1H), 3.75 (t, \(J = 6.7\) Hz, 2H), 3.57 (t, \(J = 6.3\) Hz, 2H), 2.27 (s, 3H), 2.26 (s, 3H), 2.23 (s, 3H), 1.96-1.90 (m, 2H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \(\delta\) 173.6, 158, 138.1, 137.5, 135.5, 130.5, 129.9, 125.6, 114.8, 47.7, 41.6, 31.2, 19.8, 19.4, 12.5, 11.0.

The alkyl chloride \textit{19} (0.85g, 2.2 mmol) was dissolved in 7mL of DMF. Sodium azide (0.4g, 6.6 mmol) was then added and the reaction mixture was heated to 80°C (oil bath temperature). After 16 hrs the mixture was allowed to cool to RT and 30mL water was added. The reaction mixture was then extracted with EA (2 x 50mL). The combined organic layers were washed with brine (100mL), dried with MgSO\textsubscript{4}, filtered and concentrated \textit{in vacuo}. This gave azide \textit{10} as a yellow foam (0.80g, 92%) which was used without further purification.

\textbf{(3-Azidopropyl)(3,5-dimethyl-4-isoxazolylsulfonyl)(3,4-xylyl)amine 10.} TLC Rf = 0.46 (30% EA/70% hexanes); IR (ATR) 2938, 2358, 2096, 1587, 1346, 1180 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.09 (d, \(J = 8.0\) Hz, 1H), 6.93 (d, \(J = 2.1\) Hz, 1H), 6.83 (dd, \(J = 10.2, 2.2\) Hz, 1H), 3.68 (t, \(J = 6.6\) Hz, 2H), 3.38 (t, \(J = 6.6\) Hz, 2H), 2.25-2.24 (m, 6H), 2.22 (s,
3H), 2.09 (s, 3H), 1.79-1.72 (m, 2H); 13C NMR (100 MHz, CDCl3) δ 173.6, 158, 138.2, 137.6, 135.4, 130.5, 129.8, 125.6, 114.9, 48.4, 47.6, 27.8, 19.7, 19.4, 12.5, 11.0.

The azide 10 (1.00g, 2.75 mmol) was dissolved in 20mL of THF. Water (4mL) was then added followed by triphenyl phosphine (0.79g, 3.03 mmol). After 16 hrs at RT the solvent was evaporated and excess triphenyl phosphine was removed by passing the residue through a short plug of silica gel (20% EA/80%hexanes). This provided the amine product containing some triphenyl phosphine oxide, which was used without further purification.

Biotin (1.32g, 5.4 mmol), EDCl (0.84g, 5.4 mmol) and diisopropylethylamine (0.95mL, 5.4 mmol) were dissolved in 27 mL of DMF and stirred for 10 min. The crude amine (0.91g) was then added to the reaction mixture. After 16 hrs at RT brine (30mL) was added and the reaction mixture was extracted with EA (3 x 30mL). The combined organic layers washed with water (1 x 30mL) and brine (2 x 30mL), dried (MgSO4), filtered and concentrated. The residue was purified using silica gel chromatography (15% MeOH/85% DCM) to provide the amide 11 (biotin-P13) as a waxy yellow solid (0.30g, 20%).

4-[(3aS,4S,6aR)-2-Oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl]-N-(3-[N-(3,4-dimethylphenyl)3,5-dimethyl-1,2-oxazole-4-sulfonamido]propyl)butanamide 11 (biotin-P13). TLC Rf = 0.32 (10% MeOH/90% DCM); IR (ATR) 3208, 2923, 2359, 2341, 1696, 1641, 1342, 1116, 688 cm−1; 1H NMR (400 MHz, CDCl3) δ 7.14 (d, J = 8.4 Hz, 1H), 7.00–6.99 (m, 1H), 6.92 (dd, J = 8.0, 2.2 Hz, 1H), 6.47 (bs, 1H), 5.55 (bs, 1H). 5.23 (bs, 1H), 4.42-4.39 (m, 1H), 4.23-4.20 (m, 1H), 3.61 (t, J = 5.1 Hz, 2H), 3.17-3.12 (m, 3H), 2.87 (dd, J = 12.7, 4.9 Hz, 1H), 2.63 (d, J = 12.7 Hz, 1H), 2.27 (s, 3H), 2.25 (s, 3H), 2.21
(s, 3H), 2.08 (t, J = 7.4 Hz, 3H), 1.99 (s, 3H), 1.71-1.48 (m, 6H), 1.39-1.31 (m, 2H); 13C NMR (100 MHz, CDCN) δ 174.7, 173.7, 9, 163.9, 158.8, 138.8, 138.3, 136.5, 131.0, 130.7, 127.0, 115.5, 62.3, 60.7, 56.3, 48.7, 41.1, 36.9, 36.3, 29, 28.9, 28.8, 26.4, 19.7, 19.4, 12.8, 11.1; HRMS (ESI+) m/z calcd for C26H37N5O5S2 [M + Na]+: 586.2134, found: 586.2119.

The azide 12 (1.00g, 2.75 mmol) was dissolved in 20mL of THF. Water (4mL) was then added followed by triphenyl phosphine (0.79g, 3.03 mmol). After 16 hrs at RT the solvent was evaporated and excess triphenyl phosphine was removed by passing the residue through a short plug of silica gel (20% EA/80%hexanes). This provided the amine product containing some triphenyl phosphine oxide, which was used without further purification.

4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid (BODIPY Acid 13) (0.043g, 0.148 mmol) was dissolved in DMF (2mL) and HATU (0.112g, 0.296 mmol) was added. After 5 min the amine from the previous step (0.050g, 0.148 mmol) was added followed by addition of diisopropylethylamine (0.05mL, 0.296 mmol). The reaction mixture was stirred at RT for 16 hrs, and was then quenched by addition of sat. NH4Cl (10mL) and then extracted with DCM (3 x 5mL). The combined organic layers washed with water (1 x 5mL) and brine (2 x 5mL), dried (MgSO4), filtered and concentrated. Purification of the residue by column chromatography (30% DCM/EA) yielded the amide 14 (BODIPY-P13) as dark red crystals (0.078g, 87%).
12-[(3-[N-(3,4-Dimethylphenyl)3,5-dimethyl-1,2-oxazole-4-sulfonamido]propyl)carbamoyl]ethyl]-2,2-difluoro-4,6-dimethyl-1λ⁵,3-diaza-2-boratricyclo[7.3.0.0³,⁷]dodeca 1(12), 4,6,8,10-pentaen-1-ylium-2-uide 14 (BODIPY-P13). mp = 175-177°C; TLC Rf = 0.68 (30% DCM/EA); ¹H NMR (400 MHz, CDCl₃) δ 7.08 (s, 1H), 7.06 (s, 1H), 6.91 (s, 1H), 6.85 (d, J = 3.7 Hz, 1H), 6.81 (d, J = 7.9 Hz, 1H), 6.27 (d, J = 3.7 Hz, 1H), 6.13 (s, 1H), 5.99 (bs, 1H), 3.57 (t, J = 6.4 Hz, 2H), 3.33-3.24 (m, 4H), 2.62 (t, J = 7.5 Hz, 2H), 2.56 (s, 3H), 2.26 (s, 3H), 2.24 (s, 6H), 2.22 (s, 3H), 2.06 (s, 3H), 1.61-1.54 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 173.4, 171.8, 160.3, 158, 157.4, 143.9, 138, 137.4, 135.3, 135.1, 133.3, 130.4, 129.9, 128.1, 125.7, 123.8, 120.4, 117.4, 115, 47.8, 36, 35.9, 27.8, 24.8, 19.8, 19.4, 14.9, 12.5, 11.3, 11.0.

4-Phenoxy aniline 15 (1.00g, 5.4 mmol) was dissolved in 15mL DMF and cooled to 0°C. Pyridine (0.90mL, 10.8 mmol) was then added. After 10 min, 3,5-dimethyl-isoxazole-4-sulfonyl chloride 1 (1.16g, 5.94 mmol) was added in portions over 15 min. After the addition was complete the ice bath was removed and the reaction mixture was allowed to warm to RT. After 16 hrs the reaction was quenched by adding 1M HCl until the pH remained below 2 (~15mL). The reaction mixture was then extracted with EA (2 x 20mL). The combined organic layers were washed with 1M HCl (2 x 20mL) and brine (2 x 20mL), dried (MgSO₄) and concentrated. The residue was purified using silica gel chromatography (40% EA/60% hexanes) that provided sulfonamide 16 as an off-white powder (1.58g, 91%).
(3,5-Dimethyl-4-isoxazolylsulfonyl)(p-phenoxyphenyl)amine 16 (P052). mp = 106-109°C; TLC Rf = 0.30 (50% EA/hexanes); 1H NMR (400 MHz, CDCl3) δ 7.35 (t, J = 7.5 Hz, 2H), 7.14 (t, J = 7.4 Hz, 1H), 7.05 (d, J = 7.4, 2H), 6.98 (d, J = 8.3 Hz, 2H), 6.93 (d, J = 9.0 Hz, 2H), 6.44 (bs, 1H), 2.42 (s, 3H), 2.29 (s, 3H); 13C NMR (100 MHz, CDCl3) δ 173.9, 157.6, 156.6, 156.5, 129.9, 129.8, 126, 123.9, 119.4, 119.2, 115.2, 12.5, 10.8; Anal. Calcd for C17H16N2O4S: C, 59.29; H, 4.68; N, 8.13. Found: C, 59.23; H, 4.78; N, 8.17.

The sulfonamide 16 (0.2g, 0.58 mmol) and K2CO3 (0.24g, 1.74 mmol) were suspended in 25mL of MeCN and stirred for 15 min at rt. 3-Chloropropyl p-toluenesulfonate (0.29g, 1.16 mmol (prepared as described in (White, 1993) was then added and the reaction mixture warmed to reflux. After 20 hrs the reaction was allowed to cool to RT and 30mL water was added. The reaction mixture was the extracted with EA (3 x 30mL). The combined organic extracts were washed with brine (100mL), dried (MgSO4), filtered and concentrated. Purification of the residue using silica gel chromatography (50% EA/50% hexanes) gave alkyl chloride 17 as an off-white powder (0.190g, 77%).

(3-Chloropropyl)(3,5-dimethyl-4-isoxazolylsulfonyl)(p-phenoxyphenyl)amine 17 (P059). mp = 95-98°C; TLC Rf = 0.21 (50% DCM/hexanes); 1H NMR (400 MHz, CDCl3) δ 7.38 (t, J = 7.8 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 7.11 (d, J = 8.8 Hz, 2H), 7.01 (d, J = 7.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 1H), 3.77 (t, J = 6.8 Hz, 2H), 3.59 (t, J = 6.3 Hz, 2H), 2.32 (s, 3H), 2.15 (s, 3H), 2.00-1.94 (m, 2H); 13C NMR (100 MHz, CDCl3) δ 173.7, 157.9, 156, 132.5, 130.2, 130, 124.3, 119.5, 118.9, 114.7, 47.9, 41.5, 31.3, 12.5, 11.0; Anal. Calcd for C20H21ClN2O4S: C, 57.07; H, 5.03; N, 6.66. Found: C, 56.98; H, 5.10; N, 6.60.
The alkyl chloride 17 (0.100g, 0.24 mmol) was dissolved in 1mL of DMF. Sodium azide (0.050g, 0.71 mmol) was then added and the reaction mixture was warmed to 80°C. After 16 hrs the reaction mixture was allowed to cool to RT and 10mL water was added. The reaction mixture was extracted with EA (3 x 5mL). The combined organic extracts were washed with brine (10mL), dried (MgSO₄), filtered and concentrated. This provided the azide 18 a tan solid (0.090g, 87%).

(3-Azidopropyl)(3,5-dimethyl-4-isoxazolylsulfonyl)(ρ-phenoxyphenyl)amine 18 (P062). mp = 72-76°C; TLC Rf = 0.63 (30% EA/70% hexanes); IR (ATR) 3076, 2935, 2091, 1585, 1484, 1345 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.37 (t, J = 7.7 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 7.11 (d, J = 8.8 Hz, 2H), 7.01 (d, J = 8.2 Hz, 2H), 6.97 (d, J = 8.7 Hz, 2H), 3.71 (t, J = 6.7 Hz, 2H), 3.34 (t, J = 6.6 Hz, 2H), 2.31 (s, 3H), 2.15 (s, 3H), 1.79-1.72 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 173.6, 157.9, 157.8, 156, 132.4, 130.2, 130, 124.3, 119.5, 118.9, 114.7, 48.4, 47.8, 27.9, 12.5, 11.0; Anal. Calcd for C₂₇H₂₁N₅O₄S: C, 56.19; H, 4.95; N, 16.38. Found: C, 56.15; H, 4.87; N, 16.45.

The azide 18 (0.55 g, 1.29 mmol) was dissolved in 10mL of THF and water (2mL) was added followed by triphenyl phosphine (0.38g, 1.42 mmol). After 16 hrs at RT the solvent was evaporated and the residue was put through a plug of silica gel (20% EA/80%hexanes).
This gave the amine product containing small amounts of triphenyl phosphine oxide. This amine (0.05g, 0.12 mmol) was dissolved in 0.5mL of DCM and cooled to 0°C. Pyridine (0.06mL, 0.07 mmol) was then added, followed by acetic anhydride (0.013mL, 0.14 mmol). After 3 hrs at RT 5mL water was added and the reaction mixture was extracted with DCM (3 x 5mL). The combined organic extracts were washed with sat. aq. NaHCO3 (5mL) and brine (5mL), dried (MgSO4), filtered and concentrated. Purification of the residue using silica gel chromatography (10% MeOH/DCM) provided acetamide 19 as a white foam (0.040g, 73%).

1-(3-[(3,5-Dimethyl-4-isoxazolylsulfonyl)(p-phenoxyphenyl)amino]propylamino)-1-ethanone 19 (P070). TLC Rf = 0.44 (100% EA); IR (ATR) 3265, 3097, 2936, 1629, 1502, 1343, 1246 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.36 (t, J = 7.6 Hz, 2H), 7.16 (t, J = 7.4 Hz, 1H), 7.36 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 7.8 Hz, 2H), 6.95 (d, J = 8.8 Hz, 1H), 3.67 (t, J = 6.3 Hz, 2H), 3.36 (q, J = 6.2 Hz, 2H), 2.30 (s, 3H), 2.14 (s, 3H), 1.99 (s, 3H), 1.69-1.62 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 173.5, 170.5, 157.9, 157.7, 156, 132, 130.3, 130, 124.3, 119.6, 118.9, 114.8, 47.9, 36, 27.5, 23.2, 12.5, 11.0; Anal. Calcd for C₂₂H₂₅N₃O₅S: C, 59.58; H, 5.68; N, 9.47. Found: C, 59.63; H, 5.74; N, 9.26.

4-Benzylaniline (0.5g, 2.73 mmol) was dissolved in 10mL of DMF and cooled to 0°C. Pyridine (0.44mL, 5.46 mmol) was then added. After 10 min, 3,5-dimethyl-isoxazole-4-sulfonyl chloride 1 (0.59 g, 3 mmol) was added in portions over 15 min. After 16 hrs the reaction was quenched by adding 1M HCl until the pH was maintained below 2. The reaction mixture was then taken up in EA (50mL) and washed with 1M HCl (2 x 20mL)
and brine (2 x 20mL). The organic layer was then dried (MgSO₄), filtered and concentrated. The residue was purified using silica gel chromatography (10% EA/90% hexanes) to obtain sulfonamide 20 as a light brown foam (0.482g, 47%).

(3,5-Dimethyl-4-isoxazolysulfonyl)(p-benzylphenyl)amine 20 (P053). mp = 111-115°C; IR (ATR) 3227, 2951, 1588, 1490, 1337, 1179 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.28 (t, J = 7.2 Hz, 2H), 7.20 (t, J = 7.3 Hz, 1H), 7.14-7.11 (m, 4H), 6.99 (d, J = 8.4 Hz, 2H), 6.8 (bs, 1H), 3.94 (s, 2H), 2.39 (s, 3H), 2.25 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 173.9, 157.6, 140.4, 140.1, 133.2, 130, 128.8, 128.6, 126.3, 123.6, 115.3, 41.3, 12.5, 10.7; Anal. Calcd for C₁₈H₁₈N₂O₃S: C, 63.14; H, 5.30; N, 8.18. Found: C, 63.19; H, 5.27; N, 7.99.

![Structure of sulfonamide 20](image)

The sulfonamide 16 (0.2g, 0.58 mmol) and K₂CO₃ (0.18g, 1.28 mmol) were suspended in 2.5 mL of MeCN and stirred for 15 min at RT. 3-Chloropropanol (0.082g, 0.087 mmol) was then added and the reaction mixture was heated to reflux. After 20 hrs the reaction mixture was allowed to cool to RT and 30mL water was added. The reaction mixture was extracted with EA (3 x 10mL) and the combined organic layers were washed with brine (20mL), dried (MgSO₄), filtered and concentrated. Purification of the residue using silica gel chromatography (10% EA/90% hexanes) yielded alcohol 21 as a white powder (0.09g, 39%).

3-[{(3,5-Dimethyl-4-isoxazolysulfonyl)(p-phenoxyphenyl)amino]propanol 21 (P129). TLC Rf = 0.90 (50% EA/50% hexanes); ¹H NMR (400 MHz, CDCl₃) δ 7.37 (t, J = 7.6 Hz, 2H), 7.17 (t, J = 7.4 Hz, 1H), 7.12 (d, J = 9.1 Hz, 2H), 6.96 (d, J = 8.9 Hz, 2H), 3.79-3.75
(m, 4H), 2.33 (s, 3H), 2.16 (s, 3H), 1.72-1.66 (m, 4H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 173.5, 157.8, 157.7, 156.1, 132.4, 130.4, 130, 124.2, 119.5, 118.9, 114.9, 58.8, 47.3, 30.7, 12.6, 11.1; Anal. Calcd for C$_{20}$H$_{22}$N$_2$O$_5$S: C, 59.69; H, 5.51; N, 6.96. Found: C, 59.61; H, 5.63; N, 6.79.

The sulfonamide 16 (0.2g, 6.42 mmol) and K$_2$CO$_3$ (0.24g, 1.74 mmol) were dissolved in 20 mL of MeCN and stirred for 15 min at RT. 1-Bromobutane (0.12mL, 1.16 mmol) was then added and the reaction was heated to 80°C. After 20 hrs, 30 mL water was added and reaction mixture was extracted with EA (3 x 10mL). The combined organic layers were washed with brine (50mL) dried (MgSO$_4$), filtered and concentrated. Purification of the residue with silica gel chromatography (10% EA/90% hexanes) gave sulfonamide 22 as an off-white foam (0.19g, 83%).

**N-Butyl(3,5-dimethyl-4-isoxazolysulfonyl)(p-phenoxyphenyl)amine 22 (P058).** TLC R$_f$ = 0.61 (30% EA/70% hexanes); IR (ATR) 3065, 2951, 2868, 1588, 1503, 1340, 1180 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) δ 7.41 (t, $J$ = 7.6 Hz, 2H), 7.19 (t, $J$ = 7.4 Hz, 1H), 7.12 (d, $J$ = 8.9 Hz, 2H), 7.04 (d, $J$ = 7.7 Hz, 2H), 6.98 (d, $J$ = 8.9 Hz, 2H), 3.63 (t, $J$ = 6.8 Hz, 2H), 2.32 (s, 3H), 2.18 (s, 3H), 1.50-1.34 (m, 4H), 0.92 (t, $J$ = 7.2 Hz, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 173.4, 157.9, 157.6, 156.2, 132.6, 130.4, 130, 124.1, 119.4, 118.8, 115.0, 50.1, 30.2, 19.5, 13.5, 12.5, 11.0; Anal. Calcd for C$_{21}$H$_{24}$N$_2$O$_4$S: C, 62.98; H, 6.04; N, 6.99. Found: C, 62.90; H, 6.14; N, 6.85.
Alkyl chloride 17 (0.70g, 1.67 mmol) was dissolved in DMF (15mL) and NaCN (0.25g, 5 mmol) was added. The reaction was then heated to 85°C (oil bath temperature). After 18 hrs the reaction mixture was allowed to cool to RT and poured into water (50mL). The resulting mixture was extracted with EA (3x 30mL). The combined organic extracts were washed with water (50mL) and brine (2 x 50mL), dried (MgSO4), filtered and concentrated. The residue was purified using silica gel chromatography (30% EA/70% hexanes) to obtain nitrile 23 as an off-white crystalline solid (0.62g, 91%).

4-[(3,5-Dimethyl-4-isoxazolylsulfonyl)(p-phenoxyphenyl)amino]butyronitrile 23 (P075). mp = 78-83°C; TLC Rf = 0.32 (30% EA/70% hexanes); IR (ATR) 3384, 3225, 2938, 2246, 1732, 1586, 1487 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 7.41 (t, J = 7.6 Hz, 2H), 7.20 (t, J = 7.4 Hz, 1H), 7.13 (d, J = 8.9 Hz, 2H), 7.04 (d, J = 7.7 Hz, 2H), 6.99 (d, J = 8.9 Hz, 1H), 3.76 (t, J = 6.5 Hz, 2H), 2.51 (t, J = 7.3 Hz, 2H), 2.33 (s, 3H), 2.16 (s, 3H), 1.93-1.87 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 173.8, 158.1, 157.8, 155.9, 132, 130, 124.4, 119.7, 118.9, 118.7, 114.5, 49.2, 24.5, 14.4, 12.5, 11.0; Anal. Calcd for C₂₁H₂₁N₃O₄S: C, 61.30; H, 5.14; N, 10.21. Found: C, 61.25; H, 5.17; N, 10.06.

**Binding Measurements and Anisotropy**

Recombinant PP5-His₆ at the indicated concentrations was incubated on ice in 100 mM NaCl, 20 mM HEPES pH 8.0, 1% glycerol, 0.2 mM tris(2-carboxyethyl) phosphine (TCEP) and 0.5 mM MnCl₂ with 10 nM BODIPY-labeled P13 in 2% DMSO for 30 min in opaque black 96 well plates (Corning). Uncalibrated fluorescence anisotropy was then
measured using a SpectraMax i3 equipped with fluorescein anisotropy module (Molecular Devices). Curves were fit to a one-site binding equation using GraphPad Prism version 9.5.0. $y = y_0 + A \times \frac{x}{K_d + x}$ where $y$ is measured uncalibrated anisotropy, $y_0$ is the $y$ intercept, $A$ is the amplitude of the curve, $x$ is the concentration of PP5 used, and $K_d$ is the measured dissociation constant. Data are presented as mean ± SEM.

**PP5 Phosphatase Activity and Inhibition Assay**

The phosphatase activity of the recombinant PP5-His$_6$ was measured using the PiPer™ Phosphate Assay Kit (Thermo Fisher Scientific) as described in the manufacturer’s protocol. Standard curve with linear fit line was created from 0-1nM P$_i$ final concentration reactions. 1nM of PP5-His$_6$ was added to each reaction with indicated amounts of custom synthesized substrate phospho-S211-glucocorticoid receptor (PhosS211-GR) peptide (see Key Resources Table S2) as specific substrate (Thermo Fisher Scientific). Reactions were run in triplicate and incubated at 37°C for over 10min. Reaction was also performed in the presence of different amounts (100-1200 nM) of PP5 inhibitors (P5, P13 and P053). Enzyme kinetics were calculated and plotted using Lineweaver Burk plot and web-based tool (https://www.aatbio.com/tools/ic50-calculator) for calculating IC$_{50}$ and (https://bioinfo-abcc.ncifcrf.gov/IC50_Ki_Converter/index.php) for converting IC$_{50}$ to Ki values for inhibitors of enzyme activity and ligand binding (Cer, et al., 2009).

**Flow Cytometric Analysis**

Fluorescence-activated cell sorting (FACS) analysis was performed according to the protocol in the Annexin V:FITC kit (Bio-Rad). In brief, cells were plated in 10cm dish at
0.5 × 10^6 and incubated at 37°C for 18hrs. Cells were subsequently treated with compound P053 at the indicated concentrations for 18hrs. Cells were trypsinized, collected and washed once with 1x binding buffer (included in the kit). Propidium iodide was added, then the cells were immediately run on a Becton Dickinson LSRFortessa instrument (BD Biosciences). Data were analyzed using FlowJo software version 10.7.1 for Windows (BD Biosciences).

**Biotin-P5 and biotin-P13 Pulldown**

HEK293 cells were transiently transfected with PP5-FLAG or active site point mutants and protein lysate extracted. Lysate was incubated with 0.01-10µM biotin-P5 or biotin P-13 as indicated at 4°C for 1hr then added to streptavidin-conjugated agarose and incubated at 4°C for 1hr. Following three washes with fresh extraction buffer bound proteins were eluted in 5x Laemmli buffer and analyzed by Western blot. Competition experiment with P053 was conducted with protein lysate from untreated 786-O cells. Lysate was incubated with 1µM biotin-P5 or biotin-P13 for 1hr followed by competition with 1µM P053 at 4°C for 30min This was then incubated with streptavidin-conjugated agarose at 4°C for 1hr prior to washing 3x with fresh extraction buffer and elution in 5x Laemmli buffer. Samples were run by SDS PAGE, transferred to nitrocellulose membrane, and analyzed by Western blot.

**Quantification, statistical analysis and reproducibility**

The data presented are representative of three biological replicates, unless otherwise specified. All statistics were performed using GraphPad Prism version 9.5.0 for Windows
(GraphPad Software, https://www.graphpad.com). Statistical significance was ascertained between individual samples using a parametric unpaired $t$-test. Significance is denoted by asterisks in each figure: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$. Error bars represent the standard deviation for three independent experiments, unless otherwise indicated.
<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit anti-FLAG tag</td>
<td>Thermo Scientific</td>
<td>Cat# PA1-984B; RRID:AB_347227</td>
</tr>
<tr>
<td>Rabbit anti-HA tag (C29F4)</td>
<td>Cell Signaling Technology</td>
<td>Cat# 3724; RRID:AB_1549585</td>
</tr>
<tr>
<td>Mouse anti-6x-His epitope tag (HIS.H8)</td>
<td>Thermo Scientific</td>
<td>Cat# MA1-21315; RRID:AB_557403</td>
</tr>
<tr>
<td>Mouse anti-GAPDH (1D4)</td>
<td>Enzo Life Sciences</td>
<td>Cat# ADI-CSA-335; RRID:AB_10617247</td>
</tr>
<tr>
<td>Rabbit anti-PP5</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2289; RRID:AB_2168757</td>
</tr>
<tr>
<td>Mouse anti-PP5 (2E12)</td>
<td>Abcam</td>
<td>Cat# ab123919; RRID:AB_10976136</td>
</tr>
<tr>
<td>Rabbit anti-FADD</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2782; RRID:AB_2100484</td>
</tr>
<tr>
<td>Rabbit anti-phos-Ser194-FADD</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2781; RRID:AB_2100485</td>
</tr>
<tr>
<td>Rabbit anti-RIPK1</td>
<td>Cell Signaling Technology</td>
<td>Cat# 3493; RRID:AB_2305314</td>
</tr>
<tr>
<td>Rabbit anti-phos-Ser161-RIPK1</td>
<td>Invitrogen</td>
<td>Cat# PA5-105640; RRID:AB_2817068</td>
</tr>
<tr>
<td>Rabbit anti-phos-Ser166-RIPK1</td>
<td>Cell Signaling Technology</td>
<td>Cat# 44590; RRID:AB_2799268</td>
</tr>
<tr>
<td>Rabbit anti-TSC2</td>
<td>Cell Signaling Technology</td>
<td>Cat#4308; RRID:AB_10547134</td>
</tr>
<tr>
<td>Rabbit anti-phos-Ser473-Akt</td>
<td>Cell Signaling Technology</td>
<td>Cat#4060; RRID:AB_2315049</td>
</tr>
<tr>
<td>Antibody Description</td>
<td>Manufacturer</td>
<td>Cat#/RRID</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Mouse anti-Akt</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2967; RRID:AB_331160</td>
</tr>
<tr>
<td>Rat anti-Hsp90 (16F1)</td>
<td>Enzo Life Sciences</td>
<td>Cat# ADI-SPA-835; RRID:AB_11181205</td>
</tr>
<tr>
<td>Rabbit anti-phos-Ser13-Cdc37 (EPR4979)</td>
<td>Abcam</td>
<td>Cat# ab108360; RRID:AB_10859480</td>
</tr>
<tr>
<td>Rabbit anti-Cdc37</td>
<td>StressMarq Biosciences</td>
<td>Cat# SPC-142; RRID:AB_2570605</td>
</tr>
<tr>
<td>Rabbit anti-GR (D6H2L)</td>
<td>Cell Signaling Technology</td>
<td>Cat# 12041; RRID:AB_2631286</td>
</tr>
<tr>
<td>Mouse anti-GR (D4X9S)</td>
<td>Cell Signaling Technology</td>
<td>Cat# 47411; RRID:AB_2799324</td>
</tr>
<tr>
<td>Rabbit anti-phospho-GR S211</td>
<td>Cell Signaling Technology</td>
<td>Cat# 4161; RRID:AB_2155797</td>
</tr>
<tr>
<td>Rabbit anti-cleaved-PARP</td>
<td>Cell Signaling Technology</td>
<td>Cat# 5625; RRID:AB_10699459</td>
</tr>
<tr>
<td>Rabbit anti-caspase-3</td>
<td>Cell Signaling Technology</td>
<td>Cat# 9665; RRID:AB_2069872</td>
</tr>
<tr>
<td>Rabbit anti-cleaved caspase-3</td>
<td>Cell Signaling Technology</td>
<td>Cat# 9664; RRID:AB_2070042</td>
</tr>
<tr>
<td>Rabbit anti-cleaved caspase-7</td>
<td>Cell Signaling Technology</td>
<td>Cat# 9491; RRID:AB_2068144</td>
</tr>
<tr>
<td>Mouse anti-caspase-9</td>
<td>Cell Signaling Technology</td>
<td>Cat# 9508; RRID:AB_2068620</td>
</tr>
<tr>
<td>Mouse anti-caspase-8</td>
<td>Cell Signaling Technology</td>
<td>Cat# 9746; RRID:AB_2275120</td>
</tr>
<tr>
<td>Rabbit anti-cleaved caspase-8</td>
<td>Cell Signaling Technology</td>
<td>Cat# 9496; RRID:AB_561381</td>
</tr>
<tr>
<td>Rabbit anti-PP2A C Subunit</td>
<td>Cell Signaling Technology</td>
<td>Cat# 2038; RRID:AB_2169495</td>
</tr>
<tr>
<td>Rabbit anti-VHL</td>
<td>Cell Signaling Technology</td>
<td>Cat# 68547; RRID:AB_2716279</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Anti-mouse secondary</td>
<td>Cell Signaling Technology</td>
<td>Cat# 7076; RRID:AB_330924</td>
</tr>
<tr>
<td>Anti-rabbit secondary</td>
<td>Cell Signaling Technology</td>
<td>Cat# 7074; RRID:AB_2099233</td>
</tr>
<tr>
<td>Bacterial and Virus Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>EMD Millipore</td>
<td>Cat# 69450</td>
</tr>
<tr>
<td>DH5-alpha Electrocompetent E coli</td>
<td>Goldbio</td>
<td>Cat# CC-203</td>
</tr>
<tr>
<td>Biological Samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemicals, Peptides, and Recombinant Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC261</td>
<td>Abcam</td>
<td>Cat# ab145189</td>
</tr>
<tr>
<td>SNX-2112</td>
<td>Duke University; Dr. Timothy Haystead (Barrott and Haystead, 2013)</td>
<td>CAS# 908112-43-6</td>
</tr>
<tr>
<td>Z-VAD-FMK pan-caspase Inhibitor</td>
<td>Enzo Life Sciences</td>
<td>Cat# ALX-260-020-M001</td>
</tr>
<tr>
<td>PP5 (PPP5C) Human siRNA Oligo Duplex</td>
<td>OriGene Technologies</td>
<td>Cat# SR321403; SKU# SR321403A; SKU# SR321403B; SKU# SR321403C</td>
</tr>
<tr>
<td>Universal scrambled negative control siRNA duplex</td>
<td>OriGene Technologies</td>
<td>Cat# SR30004</td>
</tr>
<tr>
<td>Compound P0-P13</td>
<td>This Paper</td>
<td></td>
</tr>
<tr>
<td>Biotin-P5</td>
<td>This Paper</td>
<td></td>
</tr>
<tr>
<td>Biotin-P13</td>
<td>This Paper</td>
<td></td>
</tr>
<tr>
<td>BODIPY-P13</td>
<td>This Paper</td>
<td></td>
</tr>
<tr>
<td>P13 derivatives</td>
<td>This Paper</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Phos-Ser211-GR peptide ([NH2]PGKETNE[pS]PWR SDLL[COOH])</td>
<td>ThermoFisher Scientific custom synthesized</td>
<td>This paper</td>
</tr>
</tbody>
</table>

**Critical Commercial Assays**

<table>
<thead>
<tr>
<th>Assay Description</th>
<th>Manufacturer</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mirus TransIT-2020</td>
<td>MirusBio</td>
<td>MIR5405</td>
</tr>
<tr>
<td>Anti-FLAG M2 affinity gel</td>
<td>Sigma-Aldrich</td>
<td>A2220</td>
</tr>
<tr>
<td>Protein G agarose</td>
<td>ThermoFisher Scientific</td>
<td>15-920-010</td>
</tr>
<tr>
<td>Pierce Anti-HA Agarose</td>
<td>ThermoFisher Scientific</td>
<td>PI26182</td>
</tr>
<tr>
<td>Ni-NTA Agarose</td>
<td>ThermoFisher Scientific</td>
<td>88221</td>
</tr>
<tr>
<td>Quick Cell Proliferation Kit Plus</td>
<td>BioVision</td>
<td>K302-500; CAS# 150849-52-8</td>
</tr>
<tr>
<td>PiPer™ Phosphate Assay Kit</td>
<td>ThermoFisher Scientific</td>
<td>P22061</td>
</tr>
<tr>
<td>ANNEXIN V:FITC assay Kit</td>
<td>BIO-RAD</td>
<td>ANNEX300F</td>
</tr>
</tbody>
</table>

**Experimental Models: Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>786-O</td>
<td>CRL-1932</td>
</tr>
<tr>
<td>A498</td>
<td>HTB-44</td>
</tr>
<tr>
<td>WT HAP-1</td>
<td>C631</td>
</tr>
<tr>
<td>PP5 HAP-1 KO</td>
<td>HZGHC003163c001</td>
</tr>
<tr>
<td>FADD HAP-1 KO</td>
<td>HZGHC002596c006</td>
</tr>
<tr>
<td>RIPK1 HAP1-KO</td>
<td>HZGHC000060c015</td>
</tr>
<tr>
<td>HEK293</td>
<td>CRL-1573</td>
</tr>
<tr>
<td>HK-2</td>
<td>CRL-2190</td>
</tr>
<tr>
<td><strong>Experimental Models: Organisms/Strains</strong></td>
<td></td>
</tr>
<tr>
<td>------------------------------------------</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Oligonucleotides</strong></th>
</tr>
</thead>
</table>

| **DNA primers** | Eurofins Genomics | See Supplemental Table S2 |

<table>
<thead>
<tr>
<th><strong>Recombinant DNA</strong></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>pcDNA3-PP5-FLAG</strong></th>
<th>(Dushukyan, et al., 2017)</th>
<th>n/a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pcDNA3-HA-RIPK1</strong></td>
<td>(Seo, et al., 2016)</td>
<td>Addgene plasmid # 78834; RRID:Addgene_78834</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------</td>
<td>-----</td>
</tr>
<tr>
<td><strong>pcDNA3-FLAG-FADD</strong></td>
<td>(Lee, et al., 2012a)</td>
<td>Addgene plasmid # 78802; RRID:Addgene_78802</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------</td>
<td>-----</td>
</tr>
<tr>
<td><strong>pcDNA3-PP5-FLAG-M309C</strong></td>
<td>(Oberoi, et al., 2016)</td>
<td>n/a</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------</td>
<td>-----</td>
</tr>
<tr>
<td><strong>pcDNA3-PP5-FLAG-W386F</strong></td>
<td>(Oberoi, et al., 2016)</td>
<td>n/a</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------</td>
<td>-----</td>
</tr>
<tr>
<td><strong>pGEX6P1-PP5</strong></td>
<td>(Oberoi, et al., 2016)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Software and Algorithms</strong></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>Biorender</strong></th>
<th><a href="https://biorender.com/">https://biorender.com/</a></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PyMOL version 2.5.4 for windows</strong></td>
<td><a href="https://pymol.org/2/">https://pymol.org/2/</a></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td><strong>GraphPad Prism version 9.5.0 for windows</strong></td>
<td>GraphPad Software, La Jolla, California, USA, <a href="http://www.graphpad.com">www.graphpad.com</a></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td><strong>FlowJo 10.7.1 for windows</strong></td>
<td><a href="https://www.flowjo.com/">https://www.flowjo.com/</a></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td><strong>Chemdraw 20.1</strong></td>
<td><a href="https://perkinelmerinformatics.com/products/research/chemdraw">https://perkinelmerinformatics.com/products/research/chemdraw</a></td>
</tr>
</tbody>
</table>
Table S2

Table S2. Primer table.

Restriction sites are highlighted in blue, mutated sequences are in red, and epitope sequences are in green. Related to Figures 2.9 and 3.13.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG-FADD-ΔDD-BamHI-F</td>
<td>TATGCGGGATCCATGGATTACAAGGATGACGACGATAAGGACGACTTCCAGGCG</td>
</tr>
<tr>
<td>FADD-ΔDD-XhoI-R</td>
<td>TATGCGCTCGAGTCAGGACGCTTCCAGGAGGT</td>
</tr>
<tr>
<td>HA-RIPK1-ΔDD-KpnI-F</td>
<td>TATGCGGGTACCATGCCATACGATGTTCGAGGTACAGGACGCTTCCAGGACGACATGTCC</td>
</tr>
<tr>
<td>RIPK1-ΔDD-XhoI-R</td>
<td>TATGCGCTCGAGTTACGTCAGACTAGTGGT</td>
</tr>
<tr>
<td>PP5-R275A-F</td>
<td>GACTTTGTGGACGCCGGCTCCCTTCTCT</td>
</tr>
<tr>
<td>PP5-R275A-R</td>
<td>AGAGAAGGAGCCGGCGTCACCACAAAGGTC</td>
</tr>
<tr>
<td>PP5-H304Q-F</td>
<td>TTCACCTTCCCTGGAGGCAACCCAAGAGACAGAACAATGAACCA</td>
</tr>
<tr>
<td>PP5-H304Q-R</td>
<td>TGGTTCATGTTGTCTGTCTTTGGTTGGCTCCAGGGAGGTGAA</td>
</tr>
<tr>
<td>PP5-R400A-F</td>
<td>TCGATCAGCAAGGCCGGCGTGAGCTGT</td>
</tr>
<tr>
<td>PP5-R400A-R</td>
<td>ACAGCTCACGCCGCCCTTGGCTGATCGA</td>
</tr>
<tr>
<td>PP5-Y451F-F</td>
<td>TCTGCCCAACTTCTGCGACCAGATG</td>
</tr>
<tr>
<td>PP5-Y451R-F</td>
<td>CATCTGGTCGCAGAAAGTTGGGGGGCAGA</td>
</tr>
</tbody>
</table>
References


Elmore S. Apoptosis a review of programmed cell death. Toxicol Pathol. 2007


Grankvist N, Amable L, Honkanen RE, Sjoholm A, Ortsater H. Serine/threonine protein phosphatase 5 regulates glucose homeostasis in vivo and apoptosis signallning in mouse


