Investigating the interactions between Wilms' tumor suppressor protein and the protein ligands par4, p53, Ciao 1 and U2AF65

by

Tristen Carla Weiss
B.Sc., University of Victoria, 2004

A Thesis Submitted in Partial Fulfillment of the Requirements of the Degree of

MASTER OF SCIENCE

In the Department of Biochemistry and Microbiology

©Tristen Carla Weiss, 2007
University of Victoria

All rights reserved. This thesis may not be reproduced in whole or in part, by photocopy or other means, without the permission of the author.
Investigating the interactions between Wilms’ Tumor Suppressor protein and the protein ligands par4, p53, Ciao 1 and U2AF65

by

Tristen Carla Weiss
B.Sc., University of Victoria, 2004

Supervisory Committee

Dr. Paul Romaniuk, (Department of Biochemistry and Microbiology)

Dr. Alisdair Boraston, (Department of Biochemistry and Microbiology)

Dr. Patrick von Aderkas, (Department of Biology)
Supervisory Committee

Dr. Paul Romaniuk, (Department of Biochemistry and Microbiology)

Supervisor

Dr. Alisdair Boraston, (Department of Biochemistry and Microbiology)

Departmental Member

Dr. Patrick von Aderkas, (Department of Biology)

Outside Member

Abstract

Wilms’ tumor suppressor protein (WT1) is a key regulatory factor involved in controlling the development and normal physiology of the genitourinary tract. Mutations within WT1 result in multiple syndromes affecting the kidney and gonads with the most severe effects being Wilms’ tumor, a pediatric kidney cancer. The WT1 protein is composed of two distinct functional domains; the amino terminus is a proline and glutamine rich regulatory domain, while the carboxyl terminus is a DNA binding domain which contains four C2H2 zinc fingers. Although the zinc finger motif is small in size, proteins containing zinc fingers are extremely diverse in their functions. The functional diversity of WT1 is exemplified through its interactions with a wide range of ligands, such as DNA, RNA and proteins. The interaction between WT1 and DNA has been well characterized, while the interactions with RNA and proteins still require intensive investigation. Recent studies have identified a diverse group of WT1 protein partners but the characterization of the protein-protein interactions has been limited and inconclusive. Therefore, the experiments conducted in this study focused on investigating the
mechanism of interaction between WT1 with the protein ligands Ciao 1, p53, par4, and U2AF65.

To identify which WT1 zinc finger(s) are critical in protein binding, a series of finger swap and deletion mutant proteins were created using site directed mutagenic PCR. The effects the mutant proteins had on the protein interactions were analyzed qualitatively using GST pulldown assays. Two different approaches were used for the GST pulldown assays. The first approach utilized bacterially expressed and purified proteins. None of the mutant WT1 proteins exhibited a decrease in protein binding in these assays. Numerous pulldown trials involving various zinc finger proteins revealed non-specific protein-protein interactions were occurring. The second approach employed in vitro translated $^{35}$S-labelled proteins. The results from these assays demonstrate a clear role for WT1zf3, and a possible role for WT1zf4 in the WT1-par4 interaction. The replacement of WT1 zinc fingers 3 and 4 with those from YY1 caused a distinct reduction in binding to par4 which was exclusive for the WT1-par4 interaction. YY1 is a transcription factor from yeast that contains four C$_2$H$_2$ type zinc fingers. A decrease in binding between the chimeric proteins WT1:YY1 and the protein partners Ciao 1 and U2AF65 was also observed, although to a much lesser extent. This difference in binding ability may indicate that the interactions between WT1 and its protein ligands involve different zinc fingers.
Table of Contents

Supervisory Committee.................................................................ii
Abstract.........................................................................................iii
Table of Contents...........................................................................v
List of Tables..................................................................................ix
List of Figures..................................................................................x
Acknowledgments...........................................................................xii

Chapter 1. Overview and Introduction............................................... 1

1.1 WT1: The Zinc Finger Protein Under Investigation.......................... 2
  1.1.1 WT1 and Disease................................................................. 2
  1.1.2 WT1 Protein and Nephron Development................................. 3
  1.1.3 WT1 Protein and Cancer...................................................... 3
  1.1.4 The WT1 Gene................................................................. 4
  1.1.5 Alternative Splicing........................................................... 4

1.2 Zinc Finger Proteins..................................................................... 8
  1.2.1 C2H2 Type Zinc Fingers...................................................... 9
  1.2.2 Ligands of Zinc Finger Proteins.......................................... 11

1.3 The Functions of WT1................................................................. 15
  1.3.1 WT1 as a Transcription Factor............................................ 15
  1.3.2 The DNA Sequence Bound Specifically by WT1.................... 16
  1.3.3 Transcriptional Regulation by WT1..................................... 18
  1.3.4 Posttranscriptional Regulation........................................... 19
1.3.5 Protein-Protein Interactions ......................................................... 21
1.4 Introduction to My Study ................................................................. 25

Chapter 2. Materials and Methods ......................................................... 29

2.1 Vector Plasmids ............................................................................ 29

2.2 Preparation of Protein Partners ....................................................... 29
  2.2.1 p53 and par4 ........................................................................... 29
  2.2.2 Preparation of Protein Partners for Cloning .................................. 30
    2.2.2.1 U2AF65 ........................................................................... 30
    2.2.2.2 Ciao 1 .......................................................................... 30
    2.2.2.3 YY1 and RACK 1 .............................................................. 31
  2.2.3 Cloning of Protein Partners ......................................................... 33

2.2.4 Screening of Cloning ................................................................. 34
  2.2.4.1 Colony PCR (RACK 1, YY1) ................................................ 34
  2.2.4.2 Restriction Enzyme Digests (U2AF65, Ciao 1, RACK 1, YY1) ....... 34

2.2.5 Overexpression and Purification of Recombinant Proteins ................. 36
  2.2.5.1 WT1 and Wx mutants, TFIILA, ZAPI and AZFI .................... 36
  2.2.5.2 Recombinant GST Protein Expression and Purification .......... 36
  2.2.5.3 Recombinant His Protein Expression and Purification .......... 39

2.3 GST Pulldown Assays with Purified Proteins .................................... 40
  2.3.1 Analysis of Pulldown Reactions ................................................ 41
    2.3.1.1 SDS-PAGE and Coomassie Staining .................................. 41
    2.3.1.2 Western Blot .................................................................. 41

2.4 Radioactive Proteins and GST Pulldown Assays ................................. 43
2.4.1 TnT PCR of pET30a:WT1(-KTS), pET30a:p43zf6-9, pET30a:WT1Δ4, pET30a:WT1Δ1 ...................................................................................................................... 43
2.4.2 Gradient TnT PCR of pET30a:YY1 ................................................................................................................................. 44
2.4.3 Finger Swap Mutagenesis TnT PCR of WT1:YY1 Chimeric Sequences ...... 44
2.4.4 TnT T7 Quick Coupled Transcription/Translation Reactions .................. 47
2.4.5 GST Pulldowns with $^{35}$S-labelled proteins ................................................................. 48

Chapter 3. Results ................................................................................................................................. 49
3.1 Introduction and Overview ................................................................................................. 49
3.2 Diagnostic Screening of Cloning ....................................................................................... 50
  3.2.1 Diagnostic Colony PCR ............................................................................................................. 50
  3.2.2 Diagnostic Restriction Enzyme Digests.............................................................................. 52
3.3 Overexpression and Purification of Recombinant Proteins........................................... 54
  3.3.1 GST Recombinant Proteins ....................................................................................................... 54
  3.3.2 His Recombinant Proteins ....................................................................................................... 54
3.4 Purified Recombinant Protein GST Pulldown Method .................................................. 57
  3.4.1 WT1 (+KTS) and Four Wx+ Site-directed Mutants .......................................................... 57
  3.4.2 WT1 (-KTS) and Three Wx- Site-directed Mutants ........................................................ 58
  3.4.3 Rationale for Further Analysis .............................................................................................. 61
  3.4.4 Screening for Potential Zinc Finger Donors .................................................................... 62
3.5 Radioactive Proteins and GST Pulldown Assays .......................................................... 66
  3.5.1 TnT PCR Reactions ......................................................................................................................... 66
  3.5.2 In vitro Transcription and Translation of $^{35}$S-labeled Proteins................................. 69
  3.5.3 Pulldowns with $^{35}$S-labelled Proteins ................................................................................. 72
3.5.3.1 Screening for Potential Zinc Finger Donors ........................................... 72

3.5.3.2 Pulldowns with WT1 Deletion Mutants and Chimeric Proteins ............... 74

Chapter 4. Discussion ......................................................................................... 79

4.1 Wx Mutants of WT1 and TFIIIAzf4-7 .......................................................... 81

4.2 TFIIIAzf4-7 ................................................................................................. 84

4.3 $^{35}$S-labeled Proteins ............................................................................ 85

4.4 Deletion Mutants and Chimeric Proteins .................................................. 86

Chapter 5. Conclusion ...................................................................................... 92

Literature Cited ................................................................................................. 94
List of Tables

Table 1. Primers used in PCR to amplify the Ciao 1, RACK 1 and YY1 cDNAs encoded in pOTB7 plasmids in preparation for cloning into pGEX plasmids.............32

Table 2. Primers used in colony PCR screening for successful cloning reactions of pGEX-4T-3:RACK 1 and pET30a:YY1..................................................35

Table 3. Restriction enzymes used in double digest screening for successful cloning reactions of pGEX-4T-3:Ciao 1, pGEX-4T-3:U2AF65, pGEX-4T-3:RACK 1 and pET30a:YY1..................................................37

Table 4. Primers used in mutagenic TnT three-step PCR reactions for the creation of the WT1:YY1 chimeric proteins W12Y34 and Y12W34.........................46

Table 5. The predicted and apparent molecular weights and fraction concentration of the recombinant proteins GST, GST-Ciao 1, GST-par4, GST-p53, GST-U2AF65, His-YY1, His-ZAP1, His-p43zf1-4 and His-p43zf6-9..........................56

Table 6. Predicted sizes of TnT PCR products for WT1 (-KTS), YY1, W12Y34, Y12W34, WT1∆1, WT1∆4 and p43 zf6-9........................................68

Table 7. The apparent and predicted molecular weights for the in vitro translated proteins WT1 (-KTS), YY1, W12Y34, Y12W34, WT1∆1, WT1∆4 and p43 zf6-9......71
List of Figures

Figure 1. The four major WT1 protein isoforms created by two alternative splice sites.. 5

Figure 2. A combination of three translation initiation sites, alternative splicing, and RNA editing results in the generation of 24 different WT1 protein isoforms. .............. 7

Figure 3. The C_2H_2 motif .................................................................................................................. 10

Figure 4. The interaction of the three zinc finger containing EGR1 (Zif268) with the major groove of DNA. .................................................................................................................. 12

Figure 5. Schematic of WT1 (+KTS) and Wx mutant zinc finger domain cDNAs created by site direct mutagenesis. .................................................................................................................. 26

Figure 6. Schematic of the W12Y34 and Y12W34 chimeric proteins. ................................. 27

Figure 7. Schematic of the three-step PCR reactions used to create the WT1:YY1 chimeric proteins.......................................................................................................................... 45

Figure 8. Identification of pET30a:YY1 plasmids by colony PCR................................. 51

Figure 9. Identification of pGEX-4T-3:U2AF65 plasmids by restriction enzyme digests.......................................................................................................................... 53

Figure 10. SDS-PAGE of GST-Ciao 1 protein fractions purified by a batch purification method.................................................................................................................. 55

Figure 11. Western blot of GST pulldown reactions between GST-p53 and WT1 (+KTS) wild type and Wx+ mutants. .................................................................................................................. 59

Figure 12. SDS-PAGE of GST pulldown reactions of WT1 (-KTS) wild type and Wx- mutants probed against GST fusion proteins Ciao 1, p53 and par4 .......... 60

Figure 13. Western blot of GST pulldown reactions between TFIIIAzf4-7 and GST-Ciao 1. .......................................................................................................................... 63
Figure 14. Western blot of GST pulldown reactions between p43zf1-4, p43zf6-9 and GST-Ciao 1........................................................................................................ 64

Figure 15. SDS-PAGE of GST pulldown reactions between TFIIIAzf4-7 and GST-Ciao 1........................................................................................................ 65

Figure 16. Product of TnT PCR amplification of WT1 (-KTS). ........................................ 67

Figure 17. Autoradiogram of in vitro transcription and translation reactions of WT1 (-KTS) and YY1. ......................................................................................... 70

Figure 18. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, p43zf6-9 and GST fusion proteins Ciao 1, RACK1 and GST............................. 73

Figure 19. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and GST-Ciao 1............................................. 75

Figure 20. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and GST-U2AF65............................................ 76

Figure 21. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and GST-par4.............................................. 78

Figure 22. Possible interactions between WT1 and its different protein ligands........... 89
Acknowledgments

There are many people that I would like to thank for making my graduate studies a memorable experience. First of all I would like to thank Paul Romaniuk for giving me the opportunity as a graduate student, for his patient and thorough explanations, and for his guidance throughout my course of study. I would also like to thank all past and present members of the PJR lab, Cheng Yang, Heather Croft, Megumi Takiguchi, Chelsea Patrick, Lindsay Bishop, Katie Gregg, Simran Bhatia and especially Julie Foster for all their great ideas and support in and out of the lab. In addition, I would like to give a big thank you to my fellow colleagues in the Boraston lab for all their input, inspiration and laughter.

And finally, I would like to thank my family for their support and absolute confidence in me. Thanks to my parents, Carl and Dixie Weiss, for the continued push to start and finish. And to Damian, Mira and Emma for their never-ending patience and understanding.
Chapter 1. Overview and Introduction

Zinc finger proteins belong to one of the most abundant regulatory protein families found in mammals (Laity et al., 2001). Traditionally thought of as DNA-interaction modules, extensive research has revealed zinc finger proteins that interact with RNA, proteins and other small molecules (Krishna et al., 2003). The functional diversity of zinc finger proteins is exemplified by their involvement in a wide variety of cellular processes such as regulation of apoptosis, transcription and translation, cell proliferation and lipid binding (Krishna et al., 2003).

The zinc finger was first discovered two decades ago (Miller et al., 1985). Since then the interactions between zinc finger proteins and nucleic acids have been extensively studied. A compilation of studies including x-ray crystallography structures, site-directed mutagenesis and quantitative binding assays has provided a basic framework for the mechanism of these interactions. More recently, studies have been focusing on the characterization of zinc finger protein-protein interactions. The full analysis of these interactions will provide a complete understanding of the diverse functions of these complex proteins.

The present study focuses on the zinc finger protein WT1. WT1 is a tumor suppressor protein involved in controlling development and normal physiology of the genitourinary tract (Pritchard-Jones et al., 1990) and mutations within WT1 result in several syndromes afflicting the genitourinary tract (Orkin et al., 1984; van Heyningen et al., 1990; Coppes et al., 1993; Little et al., 1993; Wagner et al., 2003). WT1 has the unique ability to specifically interact with multiple types of ligands including DNA, RNA
and proteins. As with other zinc finger proteins, the nucleic acid-binding ability of this protein has been extensively studied while the protein interactions are much less understood. Further analysis will provide insight into the mechanism of interaction between this complex protein and its protein partners.

1.1 WT1: The Zinc Finger Protein Under Investigation

1.1.1 WT1 and Disease

Wilms' tumor (WT) was first described in 1899 by Max Wilms (Scharnhorst et al., 2001). It is a pediatric kidney cancer which occurs at a rate of 1 in 10,000 births, usually presenting itself in children below the age of five years (Hastie, 1994). Although both sporadic and familial forms exist, familial Wilms' tumor is exceptionally rare and occurs with a frequency of less than 1% in all cases of Wilms' tumor (Matsunaga, 1981). The majority of Wilms' tumors are caused by sporadic somatic mutations. Approximately 15% of these sporadic tumor cases are the result of mutations which cause the inactivation of the WT1 gene (Orkin et al., 1984; van Heyningen et al., 1990; Coppes et al., 1993; Little et al., 1993). It has been found that there is a predisposition to WT in association with WAGR (Wilms' tumor, aniridia, genitourinary malformations, mental retardation), Denys-Drash, Beckwith-Wiedemann, and Frasier syndromes (Wagner et al., 2003). The disease has a cure rate of 85% following a combination of surgical resection and chemotherapy (Green, 1997).
1.1.2 WT1 Protein and Nephron Development

The WT1 protein functions as a tumor suppressor and is a key regulatory factor in controlling genitourinary development and normal physiology. The development of the mammalian urogenital tract occurs in a three-step process which is dependent upon WT1 expression. The expression of WT1 results in a mesenchymal to epithelial transition (Kriedberg et al., 1993). The highest levels of WT1 gene expression occur within the embryonic kidney (Call et al., 1990) as well as several tissues of mesodermal origin, including the gonads, spleen and mesothelial linings of abdominal and thoracic cavities (Pritchard-Jones et al., 1990). This implicates WT1 in a dual role involving both development and homeostasis (Buckler et al., 1991).

1.1.3 WT1 Protein and Cancer

Wilms' tumor is derived from mesenchymal stem cells that have failed to undergo differentiation, which normally produce the epithelial portion of the nephron. The tumors are of the so-called triphasic type, meaning they are composed of a combination of stromal cells, epithelial cells and undifferentiated blastema. Mutations in the WT1 gene result in a plethora of developmental abnormalities and cancer with the most severe effects on the kidney and gonads (Hastie, 2001).

Wilms' tumor has been a continual topic of interest and research for several reasons. Research indicates that the tumor occurs due to development gone awry and therefore investigations may lead to answers in developmental processes. In addition, the tumor is found in association with other congenital abnormalities; further research may
provide some insight into possible overlapping pathways in these syndromes (Scharnhorst et al., 2001).

1.1.4 The WT1 Gene

Wilms’ tumor suppressor gene (WT1) was identified in 1990 through positional cloning and it mapped the gene to human chromosome 11p13. The transcriptional unit spans approximately 50 kilobases (kb) and consists of 10 exons. The gene encodes an mRNA transcript of 3 kb, which can undergo alternative splicing and RNA editing to yield many isoforms. The protein product has a molecular weight of approximately 50 kDa depending on the specific isoform (Call et al., 1990).

The WT1 gene product, Wilms’ tumor suppressor protein (WT1) is composed of two distinct functional domains (Haber et al., 1991). The amino terminus is a proline and glutamine rich regulatory domain, while the carboxyl terminus is a DNA binding domain which contains four C2H2 zinc fingers similar to the DNA-binding domain found in the Drosophila Krüppel protein (Discenza and Pelletier, 2004).

1.1.5 Alternative Splicing

In mammals, alternative splicing events involving exons 5 and 9 occur, which result in four distinct protein isoforms (Figure 1) (Haber et al., 1991). Splice I involves the insertion of 17 amino acids encoding exon 5, between the amino terminus and the zinc finger domain. It has been found that all other vertebrates lack exon 5 making this splicing event unique to mammals (Kent et al., 1995). The physiological significance of the presence or absence of exon 5 is an area of debate as some studies suggest it has little
Figure 1. The four major WT1 protein isoforms created by two alternative splice sites. The inclusion of exon 5 encodes 17 amino acids inserted between the proline/glutamine rich region and the C-terminal zinc finger domain. The use of alternative splice donor site at the 3' end of exon 9 inserts amino acids, KTS, between the third and fourth zinc fingers. Adapted from Hirose, 1999.
consequence to protein function, while contrasting views suggest it has a modulating role (Wang et al., 1985; Richard et al., 2001). Contained within exon 5 is a protein-protein interaction domain that allows for the association with prostate apoptosis response factor 4 (par4) (Richard et al., 2001). Furthermore, the ratio of WT1 variants containing exon 5 varies during development and between cell types, which will be discussed below. The second alternative splicing event occurs at the end of exon 9 and results in the insertion of three amino acids (KTS) between zinc fingers 3 and 4 (Haber et al., 1991). This alternative splicing event has been observed in cDNA from humans to alligators, zebrafish, mouse, marsupial mouse and chick (Kent et al., 1995). Isoforms containing the KTS insert are typically referred to as WT1 (+KTS) and those lacking the insert are referred to as WT1 (-KTS). The WT1 (-KTS) isoform has a high affinity for DNA while the incorporation of KTS creates a hinge, which disrupts the DNA binding ability of the protein (Haber et al., 1991), creating a higher affinity for RNA (Caricasole et al., 1996). The combination of RNA editing, three alternative translation initiation sites and alternative splicing results in the generation of at least 24 different protein isoforms (Figure 2) (Haber et al., 1991; Bruening and Pelletier, 1996; Scharnhorst et al., 1999).

The use of RNase protection assays determined that the most abundant isoform contains both exon 5 and the KTS insertion, while the transcript lacking both is the least common (Haber et al., 1991). The ratio of WT1 (+KTS) to WT1 (-KTS) is consistent during development (Haber et al., 1991) with a range of 1.1-1.5:1 (Hammes et al., 2001). It has been found that the fetal kidney equally expresses all four main isoforms while the adult kidney expresses the exon 5 containing isoforms with a 2-fold excess (Renshaw et al., 1997). Although the ratio of WT1 (+KTS) to WT1 (-KTS) remains relatively
Figure 2. A combination of three translation initiation sites, alternative splicing, and RNA editing results in the generation of 24 different WT1 protein isoforms. The top panel depicts the genomic organization of WT1, consisting of 10 exons. Three alternative translation initiation sites are present in exon 1. Exon 5 and the 3’ end of exon 9 (open boxes) represent alternatively spliced sequences. The translation stop site, TGA, is shown in exon 10. The bottom panel depicts the RNA editing site at amino acid 280, resulting in a switch from leucine to proline. Adapted from Discenza and Pelletier, 2004.
constant, the ratio of the exon 5 variant tends to vary depending on species, cell type, as well as developmental stage (Wagner et al., 2003). Analysis of Wilms’ tumors revealed isoform ratios consistent with those found in the fetal kidney (Renshaw et al., 1997). The fact that the WT1 isoforms play functionally important roles is evident not only in the consistency of their relative abundance in WT1-expressing tissues, but also in the high sequence conservation across species (Kent et al., 1995).

The importance of isoform ratios is perhaps most clearly demonstrated by considering Frasier syndrome. This syndrome results from a point mutation in the intron 9 splice site of one WT1 allele, causing a loss of expression of the WT1 (+KTS) isoform. The resulting change in isoform ratio is responsible for the developmental defects found in this syndrome. Therefore, although the WT1 isoforms may have some overlapping functions, there appears to be some functions that are unique and cannot be compensated for (Hammes et al., 2001).

1.2 Zinc Finger Proteins

The zinc finger is a common motif found in transcription factors and many other DNA-binding proteins (Krishna et al., 2003). It was first described as a repeating small peptide sequence containing conserved cysteine and histidine residues that coordinate tetrahedrally to a zinc ion (Miller et al., 1985). The term ‘zinc finger’ is now used to describe a diverse group of proteins. These proteins are characterized as having small protein domains that are structurally stabilized by a zinc ion, which is generally not involved in their function (Klug and Schwabe, 1995). Although the zinc finger motif is small in size, proteins containing zinc fingers are extremely diverse in their functions. In
addition to their ability to bind nucleic acids and other proteins, it has also been found that zinc fingers can bind small ligands such as lipids and may also show enzymatic properties. These various interactions implicate zinc finger proteins in many cellular processes such as cell signaling, proliferation, apoptosis, repair, replication and transcription (Krisha et al., 2003).

1.2.1 C$_2$H$_2$ Type Zinc Fingers

The C$_2$H$_2$ type of zinc finger was first characterized in the transcription factor IIIA (TFIIIA) in Xenopus laevis (Miller et al., 1985). C$_2$H$_2$ zinc finger proteins are thought to comprise up to 1% of all mammalian proteins (Hoovers et al., 1992) and are found in other eukaryotes and prokaryotes (Iuchi, 2001). The left-handed ββα-unit consists of a repeating unit of 28-30 amino acids containing two conserved cysteines and two conserved histidines (Figure 3) (Miller et al., 1985). These are arranged as two anti-parallel β strands followed by an α helix with the overall fold being stabilized by the tetrahedral coordination of the cysteines and histidines to a zinc atom (Berg, 1988; Lee et al., 1989). The classical C$_2$H$_2$ zinc finger motif consists of the pattern X$_2$-Cys-X$_{2,4}$-Cys-X$_{12}$-His-X$_{3,5}$-His (where X is any amino acid residue) (Miller et al., 1985). The motif generally occurs in tandem repeats containing 2 to 37 zinc fingers separated by a linker sequence. Approximately half the known proteins contain the linker sequence TGEKPP, which connects contiguous fingers. Mutagenesis of the linker sequences has revealed the requirement of the linker for high affinity DNA binding (Choo and Klug, 1993). Comparison studies using NMR analysis have determined that this sequence may
Figure 3. The C$_2$H$_2$ motif consists of two anti-parallel $\beta$ strands followed by an $\alpha$ helix. The overall fold of the classical C$_2$H$_2$ zinc finger is stabilized by the tetrahedral coordination of the two invariant cysteines and two histidines to a zinc atom. Internet source: http://en.wikipedia.org/wiki/Zinc_finger
contribute to the stability of the α helix by capping the C-terminal end (Laity et al., 2000).

The first detailed information regarding zinc finger-DNA interactions was revealed after solving the crystal structure of the EGR1-DNA complex (also called Zif268) (Pavletich and Pabo, 1991). EGR1 is a transcription factor containing three C$_2$H$_2$ type zinc fingers. The DNA interaction surface of nucleic acid-binding C$_2$H$_2$ zinc fingers is located within the α helix towards the N-terminal region of the two histidines. The interaction of the zinc finger protein and DNA occurs between this region of the α helix and the major groove of DNA (Figure 4) (Pavletich and Pabo, 1991). Although this structure has provided a framework for understanding the interaction with DNA, some zinc finger-DNA complexes have shown variations in docking arrangements. Despite commonly being found in transcription factors, zinc fingers can also be involved in protein-protein interactions, and interactions with RNA (Krishna et al., 2003). It has been postulated that proteins with a large number of fingers will have specific affinity for a greater number of different ligands (Iuchi, 2001).

1.2.2 Ligands of Zinc Finger Proteins

Zinc finger proteins are traditionally thought of as DNA-binding molecules. Many proteins are now found to have more than one type of ligand, interacting with DNA, RNA, proteins or a combination of several ligands. This broader range of ligands implicates proteins in a wider variety of cellular processes. Included in this class of multifunctional proteins are the zinc finger family of proteins.
Figure 4. The interaction of the three zinc finger containing EGR1 (Zif268) with the major groove of DNA. The DNA-interaction site is located within the $\alpha$ helix, towards the N-terminal region of the two histidines. Adapted from Endres et al., 2004.
As previously mentioned in section 1.2.1, TFIIIA was the first C$_2$H$_2$ zinc finger to be described (Miller et al., 1985). TFIIIA is a transcription factor involved in the developmental regulation of *Xenopus laevis* and was the first zinc finger protein found to interact with both DNA and RNA. It plays dual roles functioning as a transcription factor and by interacting with 5S RNA and transporting it from the nucleus to the cytoplasm (Guddat et al., 1990). The nucleic acid binding ability of this nine zinc finger-containing protein has been studied extensively. It has been found that specific zinc fingers are involved in binding different nucleic acids. The first three fingers are required for DNA binding while fingers 4-6 are essential for binding RNA (Clemens et al., 1993). The N-terminal six zinc fingers of TFIIIA in complex with 31 bp of 5S DNA has been crystallized. The first three fingers wrap around the major groove and contacts are made between side chains and 2-4 consecutive base pairs found mainly on the noncoding strand of DNA. TFIIIA zinc fingers 4-6 are found in a relaxed form along one side of the DNA helix. Although the $\alpha$ helix of finger 5 also makes contacts with bases in the major groove, fingers 4 and 6 appear to act as spacer elements for recognition (Nolte et al., 1998). Further *in vitro* mutational studies proposed an essential role for zinc finger 5 in DNA binding and found zinc finger 6 critical for RNA binding. Furthermore, this study also identified the specific amino acid residues within the $\alpha$ helices that are required for nucleic acid interactions (Hamilton et al., 2001). The crystal structure of TFIIIA zinc fingers 4-6 in complex with 61 bp of RNA was later solved and was consistent with the previous findings. The contacts occur between the $\alpha$ helices of TFIIIA$\alpha$4-6 and three loop regions of 5S RNA (Lu et al., 2003). Another C$_2$H$_2$ zinc finger protein thought to have dual-function binding properties is the Krüppel-TFIIIA-related protein, MOK2. The
results of \textit{in vitro} binding assays with radiolabeled RNA homopolymers and gel shift assays have suggested that the protein also has specific DNA and RNA-binding affinities. However, the exact biological functions of the protein have yet to be elucidated (Arranz \textit{et al.}, 1997).

In addition to nucleic acid binding abilities, zinc finger proteins have also been found to participate in protein-protein interactions. For example, GATA-1 is a Cys$_4$ zinc finger containing transcription factor that plays an essential role in erythroid development (Merika and Orkin, 1995). This sequence-specific DNA binding protein recognizes a locus control region with the principal motif, GATA (Merika and Orkin, 1995). Since complex DNA-protein and protein-protein interactions are responsible for specificity in gene activation, several studies have investigated the possible protein-interactions GATA-1 may be involved in. \textit{In vitro} assays determined that GATA-1 interacts with a C$_2$H$_2$ zinc finger containing transcription factor, Sp1 (Merika and Orkin, 1995). A cotransfection assay revealed that Sp1 recruits GATA-1 and forms a ternary complex. It is thought that this interaction synergistically activates transcription through a possible looping mechanism. Furthermore, GST pulldown assays confirmed that it is the zinc finger domains of both proteins that are involved in the interaction (Merika and Orkin, 1995). Additional studies employing the yeast 2-hybrid system revealed the interaction of GATA-1 and another zinc finger protein, FOG-1 (Tsang \textit{et al.}, 1997). The FOG-1 protein is a large transcriptional coactivator comprised of four classical C$_2$H$_2$ zinc fingers and five of the unusual C$_2$HC variant. Using a yeast 1-hybrid assay, Tsang and colleagues determined that the N-terminal zinc finger of GATA-1 interacts with FOG-1, while the C-terminal zinc finger is involved in DNA binding (Tsang \textit{et al.}, 1997).
Further investigations using yeast 2-hybrid assays revealed that the five C2HC variant zinc fingers of FOG-1 are responsible for its interaction with GATA-1 (Fox et al., 1999). Although the exact mechanism of action has yet to be clarified, a direct interaction of the zinc finger domains is required for normal erythroid differentiation to occur (Cantor and Orkin, 2005).

These findings do not represent isolated events as numerous studies have demonstrated other protein-protein interactions involving zinc finger domains. Other examples include, CREB/YY1 (Zhou et al., 1995), GATA-1/EKLF (Merika and Orkin, 1995), and TFIIIA (Del Rio and Setzer, 1993).

1.3 The Functions of WT1

1.3.1 WT1 as a Transcription Factor

The WT1 protein was predicted to function as a sequence-specific transcriptional regulator based upon the amino acid sequence and structural motifs predicted to form from that sequence. These motifs include amino acid composition at the amino terminus consistent with transcriptional regulation (Mitchell and Tjian, 1989) and a DNA binding domain at the carboxyl terminus containing four zinc fingers (Berg, 1990). WT1 encodes a protein with high structural similarity to the EGR (early growth response) transcription factor family (Rauscher, 1993), which is involved in regulating cell proliferation. The amino acids present in the N-terminus through to the zinc finger domain are characteristic of known regulatory domains of transcription factor proteins. There is a high concentration of serine (10.2%), proline (9.8%), glycine (9.7%) threonine (8.8%), and glutamine (7.9%), which are also found in abundance in EGR1 (also called Zif268 and
Krox24) and EGR2 (Call et al., 1990). Proline/glutamine-rich domains are a common motif in many transcription factors (Mitchell and Tjian, 1989) and high threonine/serine content has been found in other transcription factors such as Sp1 (Call et al., 1990). The N-terminal region contains domains for homodimerization, and transcriptional repression and activation, while the C-terminal domain is involved in DNA binding.

The zinc finger domain is also highly homologous to EGR1 and EGR2 (Joseph et al., 1988) and is capable of binding to the EGR recognition sequence in DNA. There is a 61% amino acid similarity between the three zinc fingers found in EGR and zinc fingers 2, 3, and 4 of WT1 (Rauscher, 1993). Despite the similarities between WT1 and EGR1, it has been found that all three zinc fingers in EGR1 are encoded by a single exon while each zinc finger in WT1 is encoded by an individual exon (Haber et al., 1991). The distinct genomic structures suggest the two proteins do not share a common evolutionary origin (Lee and Haber, 2001).

1.3.2 The DNA Sequence Bound Specifically by WT1

The presence of a proline/glutamine-rich domain and four adjacent zinc fingers led to a multitude of studies to investigate the biochemical activities of WT1 as a transcriptional regulator. Initial studies determined the DNA consensus sequences of WT1 (-KTS) to contain either (TCC)n motifs or sites that are GC-rich, such as the EGR1 and WTE sites. The TCC-repeats were identified as potential DNA-binding sites by mapping the WT1-responsive promoter, PDGF-A through DNase I footprinting and gel mobility shift assays (Wang et al., 1993b). Experiments using whole genomic PCR identified the high affinity WTE binding site (Nakagama et al., 1995). Binding site
selection using degenerate oligonucleotides identified the GC-rich EGR1 binding site (Rauscher et al., 1990). Comparison studies with EGR1 determined an optimal GC-rich DNA-binding sequence of WT1 (-KTS).

Studies analyzing the DNA binding of EGR1 and WT1 (-KTS) have revealed many similarities. The binding of EGR1 involves specific amino acids within the \( \alpha \) helical domain of each zinc finger, which interact with three bp subsites on the DNA. The same amino acids are retained in the three homologous zinc fingers of WT1 (-KTS) and are also involved in DNA binding (Hamilton et al., 1995). The DNA binding of WT1 (-KTS) has been fully characterized through studies involving site-directed mutagenesis and nitrocellulose binding assays (Borel et al., 1996). Binding site selection studies using recombinant peptides have identified a high affinity WT1 (-KTS) DNA-binding site. The DNA consensus sequence 5' GCG-TGG-GCG-TGT 3', has a dissociation constant \( (k_d) \) of 1.14 \( \times 10^{-9} \) M (Hamilton et al., 1998). Results of mutational studies on DNA sequence have identified distinct high-affinity binding sites for WT1 (-KTS) and EGR1. Although it has been found that EGR1 and WT1 (-KTS) can bind to similar DNA sequences, a fundamental difference in their molecular binding mechanisms have been identified. Using specific thermodynamic and kinetic parameters it was revealed that the interaction of WT1 (-KTS) and DNA is an entropy-driven process, while the interaction of EGR1 and DNA is driven by both entropy and enthalpy (Hamilton et al., 1998). This is consistent with X-ray crystallographic studies, which reveal that zinc finger-DNA interactions do not all conform to the previously crystallized EGR1-DNA complex. From this study it was seen that DNA subsites contacted by zinc fingers can be longer than 3 bp and overlapping. It was also found that some zinc fingers
may not be involved in the interaction with DNA, and that these contacts are not restricted to one strand of DNA (Pavletich and Pabo, 1993). Several potential binding sites for WT1 (+KTS) have been suggested, but to date nothing has been confirmed.

1.3.3 Transcriptional Regulation by WT1

The formation of fusion proteins containing WT1 and GAL4 indicated that WT1 contains domains that can independently repress or activate transcription. Co-transfection assays were performed using fusion proteins containing the yeast GAL4 transcription factor and two functional domains of WT1. The location of an activation domain was determined to be positioned at amino acids 181-250, while the repression domain was identified at amino acids 85-124 (Wang et al., 1995a). Furthermore, an experiment carried out with a fusion of GAL4 and the 17 amino acids encoded by exon 5 determined that this region alone is capable of transcriptional repression. This result suggests that this specific alternative splicing event may modify the regulatory properties of the WT1 protein (Wang et al., 1995b).

From investigating tumor-associated missense mutations, some researchers suggest that the crucial transcriptional function of WT1 is activation rather than repression. It has been found that N-terminal mutations render the protein faulty as a transcriptional activator but do not affect its ability to repress putative target promoters (English and Licht, 1999).

Studies have identified many potential target genes of WT1 such as growth factors, growth factor receptors, transcription factors, secreted or extra-cellular proteins. A number of potential target genes were identified through transient transfection assays
to be repressed by WT1, for example, *IGF-II* (Drummond *et al.*, 1992), *WT1* (Rupprecht *et al.*, 1994), *c-myc* and *bcl-2* (Hewitt *et al.*, 1995). Several of these gene targets such as *bcl-2* (Mayo *et al.*, 1999), and *c-myc* (Han *et al.*, 2004), have also been found to be activated by WT1 (-KTS). This discrepancy between activation and repression appears to depend on cell type and promoter context. Additional putative gene targets induced by WT1 (-KTS) have also been documented. These include *Hsp70, p21Cip1, aFGF* and *amphiregulin* (Lee *et al.*, 1999).

### 1.3.4 Posttranscriptional Regulation

The subnuclear expression pattern of the WT1 (+KTS) and WT1 (-KTS) isoforms suggests they are involved in distinct functions. Through confocal microscopy it can be seen that the WT1 (-KTS) isoform is localized diffusely throughout the nucleoplasm, while the WT1 (+KTS) isoform is found within 30-50 nuclear speckles (Englert *et al.*, 1995; Larsson *et al.*, 1995). Costaining of these speckles revealed colocalization of the WT1 (+KTS) isoform with small nuclear ribonuclear proteins (snRNPs), as well as splicing factors (Larsson *et al.*, 1995). The ability of WT1 (+KTS) to associate with these factors and bind to RNA with high affinity, suggests a possible involvement in posttranscriptional activities.

The nuclear speckles consist of WT1 (+KTS) and molecules implicated in mRNA splicing. U2AF65 is a constitutive splicing protein which binds to the 3' splice site of mRNA and has been shown to directly associate with WT1 (+KTS). It has also been found that the WT1 (+KTS) isoform can become directly incorporated into spliceosomes (Davies *et al.*, 1998). WT1 (+KTS) copurifies with the nuclear poly(A)^+
ribonucleoprotein (RNP) fraction which contains RNP complexes involved in the processing of pre-mRNA (Ladomery et al., 1999). Furthermore, it has recently been determined that WT1 has the ability to shuttle between the nucleus and the cytoplasm and has been found associated with actively translating polysomes (Niksic et al., 2004). Although this data suggests the involvement of WT1 (+KTS) in posttranscriptional processing of mRNA, and translation, a direct biological role has not yet been demonstrated.

In addition to its sequence-specific binding to DNA, it has been found through mutational analysis and RNase treatment assays that WT1 is capable of binding to IGF-II exonic 2 RNA (Caricasole et al., 1996). Studies using SELEX (systematic evolution of ligands by exponential enrichment), an RNA selection method, have identified three groups of RNA ligands that are specifically recognized by WT1 (Bardeesy and Pelletier, 1998). The zinc finger domain has also been implicated in the binding of RNA although conflicting studies report discrepancies in which isoform or fingers are involved. Caricasole et al., demonstrated that both the WT1 (+KTS) and WT1 (-KTS) isoforms bind RNA in a sequence-specific manner, specifically zinc finger I (Caricasole et al., 1996). Contrasting findings of Bardeesy and Pelletier suggest the WT1 (-KTS) isoform binds with a greater affinity than the WT1 (+KTS) isoform. Furthermore, truncated WT1 proteins have shown that zinc finger 1 is not detrimental for RNA binding, while the deletion of zinc finger 4 is deleterious for RNA aptamer binding (Bardeesy and Pelletier, 1998). It is interesting to note that TFIIIA from Xenopus laevis is the only other cellular factor known to act as a transcriptional regulator through the binding of both RNA and DNA. Both TFIIIA and WT1 contain Krüppel-type zinc fingers that participate in the
binding of nucleic acids (Caricasole et al., 1996) and their ability to bind DNA and RNA appear to be distinct activities involving different zinc fingers. Our lab has performed extensive investigations in the comparison of DNA and RNA binding activities of WT1 and TFIIIA with the use of site directed mutagenesis and quantitative binding assays. Although cellular RNA targets have yet to be identified, it is known that the overall RNA ligand structure is a major determinant for the binding affinity to WT1 (Zhai et al., 2001).

1.3.5 Protein-Protein Interactions

WT1 was initially characterized as a DNA-binding transcription factor, subsequent studies described its RNA-binding abilities, and most recently experimental evidence suggests WT1 has candidate protein partners. Studies have determined that WT1 has the ability to alternatively activate or repress target genes depending on physiological context. Due to the dual functionality of this protein, a simple model has been proposed: the activities of WT1 are determined by its interactions with various proteins (Johnstone et al., 1998). As previously discussed, WT1 has been found to directly interact with splice factor proteins such as U2AF65 (Davies et al., 1998). Other protein-protein interactions include WT1 dimerization, interaction with other transcription factors and several other cellular proteins (Englert, 1998). The yeast two-hybrid system has provided a screening method that allows researchers to uncover potential protein partners, some of which will be discussed below.

The interaction between WT1 and the transcription factor, p53, was identified through coimmunoprecipitation (Maheswaran et al., 1993). The coimmunoprecipitation assays revealed an in vivo interaction of WT1 and p53 within the physiological context of
baby rat kidney (BRK) cells and Wilms' tumor specimens (Maheswaran et al., 1993). The binding of the two proteins results in the alteration of their ability to transactivate their specific gene targets. p53 converts the transcriptional activation of WT1 to repression, while WT1 reduces the transcriptional repression activities of p53 and enhances p53-mediated activation. This finding further supports the function of WT1 as a transcriptional activator rather than a repressor (Maheswaran et al., 1993).

Cotransfection assays determined that the interaction of the two proteins led to the stabilization of p53 which resulted in modulation of its trans-activational properties (Maheswaran et al., 1995). The p53-interaction site was mapped to WT1 zinc fingers 1 and 2 (Maheswaran et al., 1995). Conflicting studies using deletion mutants report that it is WT1 fingers 3 and 4 that are crucial for its interaction with p53 and other p53 family members (Scharnhorst et al., 2000). Further investigations into the interaction of these two tumor suppressor proteins could potentially lead to insights into their roles in normal development and tumorigenesis.

The yeast two-hybrid system led to the discovery of another WT1-interacting protein, prostate apoptosis response factor 4 (par4, also known as PAWR) (Johnstone et al., 1996). Par4 is upregulated during apoptosis in prostate cells and functions as a transcriptional regulator. GST fusion protein-based assays have deduced that it is the zinc finger domain of WT1 which interacts with the leucine zipper region of par4 (Johnstone et al., 1996). The interaction was further examined with a cell line endogenously expressing both proteins and was confirmed by coimmunoprecipitation (Johnstone et al., 1996). Cotransfection assays using reporter constructs revealed that par4 is able to decrease WT1-mediated transcriptional activation in a dose-dependent
manner and can also enhance the transcriptional repression activity of WT1 (Johnstone et al., 1996). It is interesting to note that further studies have shown par4 can interact with the 17 amino acids (aa) introduced by alternative splicing into WT1 (Richard et al., 2001). As previously mentioned, the 17 aa insert encoded by exon 5 is mammalian-specific although its function is not entirely clear. In vitro transcription assays have suggested that the 17 aa insert imparts a transcriptional activation function (Richard et al., 2001). In vivo transfection assays have determined that par4 physically interacts with this 17 aa motif and functions as a coactivator (Richard et al., 2001). The results of these two studies pose additional questions regarding the WT1-par4 interaction. Is it possible that there are two par4-interacting sites on the WT1 protein, or is the binding site context specific?

The physical interaction of Ciao 1 and WT1 was discovered through the yeast two-hybrid assay and was confirmed through coimmunoprecipitation (Johnstone et al., 1998). Ciao 1 ("bridge" in Chinese) belongs to the β-transducin repeat or WD40 family of proteins (Johnstone et al., 1998). Structurally the WD40 proteins are very similar consisting of seven repeats which fold into a seven-bladed propeller. Although the physiological roles that these proteins play are very diverse, such as signal transduction, RNA processing, and apoptosis, a common function appears to be protein-protein interactions (Neer et al., 1994). GST fusion protein based-assays revealed that all four isoforms of WT1 interact with Ciao 1 and this interaction is specific to the C-terminal zinc finger domain of WT1 (Johnstone et al., 1998). As found with par4 (Johnstone et al., 1996), cotransfection assays resulted in Ciao 1 eliciting a dose-dependent decrease in the transcriptional activation property of WT1 (Johnstone et al., 1998). Although the
exact binding mechanism has yet to be elucidated, it has been suggested that the physical interaction of Ciao 1 binding to WT1 either causes a conformational change in the zinc finger protein or negatively interferes with its ability to interact with transcriptional machinery (Johnstone et al., 1998).

As discussed in section 1.3.4, WT1 (+KTS) colocalizes in nuclear speckles with other molecules implicated in mRNA splicing (Larsson et al., 1995). Yeast two-hybrid assays and coimmunoprecipitation were later used to identify the interaction between WT1 and the essential splicing factor U2AF65 (Davies et al., 1998). An interesting aspect of this interaction is it appears to be isoform specific; the affinity of the WT1 (+KTS) isoform for U2AF65 is much stronger than the WT1 (-KTS) isoform (Davies et al., 1998). This was initially observed in yeast two-hybrid assays and further demonstrated by titrating salt concentrations in GST binding experiments. It was found that higher salt concentrations increased the affinity of WT1 (+KTS) for U2AF65, while simultaneously decreasing the affinity of WT1 (-KTS) (Davies et al., 1998). Using truncation mutants it was revealed that although the zinc finger domain was critical for U2AF65 binding, the entire protein was required for a significant interaction (Davies et al., 1998). Further deletion mutants were constructed in an attempt to determine which zinc finger(s) may be involved in binding U2AF65. It was speculated that the (+KTS) isoform may bind RNA and splicing factors in the same manner (Davies et al., 1998) and WT1 zinc finger 1 has been suggested to be involved in RNA-binding (Caricasole et al., 1996). However, the deletion of zinc finger 1 increases the affinity of WT1 for U2AF65 in comparison with the wild type WT1 protein (Davies et al., 1998), arguing against this hypothesis.
This compilation of studies has provided researchers with an array of physiologically different WT1 protein partners, including transcriptional regulators and splicing factors. The diversity of these proteins implicates WT1 in a wide variety of cellular functions and offers some insight into the larger scope of its potential cellular roles. It is apparent from the conflicting results and inconclusive data that further exploration into the protein interactions of this complex protein is needed.

1.4 Introduction to My Study

Recent studies have provided several proteins as potential ligands of WT1. In many cases the protein-interaction site on WT1 appears to involve the zinc finger domain. Conflicting results regarding which zinc finger(s) are involved in protein binding have left the binding mechanism unresolved.

The purpose of this study was to characterize the mechanism of interaction of WT1 with the protein ligands p53, par4, Ciao 1, and U2AF65. The method of investigation involved the use of GST pulldown assays. Two sets of mutant WT1 proteins were utilized in these assays. A set of WT1 zinc finger mutants created previously by site directed mutagenesis, were employed to observe the effects on protein-protein binding. The four mutants each had a single \( \alpha \) helical region from WT1 zinc fingers 1-4 replaced with the corresponding region of TFIIBA zinc fingers 4-7 (Figure 5). The second set of mutants consisted of two chimeric proteins each containing zinc finger peptides from WT1 and YY1. One chimera was comprised of WT1 fingers 1 and 2 fused to YY1 fingers 3 and 4, the other had fingers 1 and 2 of YY1 fused to fingers 3 and 4 of WT1 (Figure 6). These mutations were expected to cause an observable decrease in
Figure 5. Schematic of WT1 (+KTS) and Wx mutant zinc finger domain cDNAs created by site direct mutagenesis. The upper panel depicts the sites of mutations, the α helical regions. The lower panel depicts the comparison of amino acid sequences of the wild type and mutant zinc fingers. The residues that are required for folding of a zinc finger are boxed in red, and the amino acid changes are shown in colour.
Figure 6. Schematic of the W12Y34 and Y12W34 chimeric proteins. The upper panel represents the four zinc finger domains from the corresponding proteins, WT1 is depicted in blue and YY1 is depicted in green. The lower panel shows the comparison of the four zinc finger amino acid sequences from both wild type WT1 (-KTS) and YY1. The boxed red region corresponds to the conserved cysteine and histidine residues in the C2H2 type zinc fingers. The boxed red and grey residues are required for proper folding of a zinc finger.
binding to protein partners, providing insight into which zinc finger(s) are critical for the protein-interactions of WT1.
Chapter 2. Materials and Methods

2.1 Vector Plasmids

Plasmid pRSET-A, which contains the cDNA of U2AF65 was kindly provided by Dr. Donald Rio (UC, Berkeley). The pGEX-2TK plasmids encoding p53 and par4 were kindly provided by Dr. Yang Shi (Harvard Medical School). The pGEX plasmids encode for fusion proteins with the GST (Glutathione S-transferase) moiety at the amino terminus. The pOTB7 plasmids containing the cDNAs of Ciao 1, YY1 and RACK1 were purchased from the American Type Culture Collection (ATCC).

2.2 Preparation of Protein Partners

2.2.1 p53 and par4

The identities of the pGEX-2TK:p53 and pGEX-2TK:par4 plasmids were confirmed by restriction enzyme digestion and DNA sequencing. Approximately 200-300 ng of the putative pGEX-2TK:par4 plasmid was digested with 5 units (U) PvuII (NEB) in a buffer recommended by the supplier for 2 hours at 37°C. A similar reaction was carried out with the putative pGEX-2TK:p53 plasmid using 5 U each of EcoRI and BamHI. The reactions were analyzed on a 0.8% agarose gel and the pattern of restriction fragments was compared to the expected fragment pattern. Plasmid isolates with the expected restriction fragment pattern were characterized further by DNA sequencing.
2.2.2 Preparation of Protein Partners for Cloning

2.2.2.1 U2AF65

A restriction enzyme digest was performed to excise the U2AF65 cDNA in preparation for cloning into the pGEX-4T-3 plasmid. Approximately 200 ng of pRSET-A:U2AF65 plasmid was digested with 5 U each of BamHI (NEB) and EcoRI (NEB) in a buffer recommended by the enzyme supplier for 1 hour at 37°C. The reaction was purified using the QIAquick PCR purification kit (QIAGEN), and an aliquot was analyzed by electrophoresis on a 1% agarose gel in 1x TBE (45 mM Tris-borate, 1 mM EDTA) buffer at 100 V. The restriction fragment with the correct size was extracted and purified using the QIAquick gel extraction kit (QIAGEN).

2.2.2.2 Ciao 1

A gradient PCR reaction was performed for amplification of the Ciao 1 cDNA sequence in preparation for cloning into the pGEX-4T-3 plasmid. A 20 μl PCR reactions containing 1.25 U of Platinum Pf× DNA polymerase (Invitrogen), 1x Pf× Amplification Buffer, 1 mM MgSO₄, 1 ng of pOTB7:Ciao 1 plasmid, 0.3 μM forward and reverse primers (Table 1), and a 0.3 mM mix of dNTPs (dATP, dGTP, dCTP, dTTP) were prepared. The reactions were amplified in a TECHNE Touchgene Gradient thermal cycler with the following conditions: an initial denaturation of 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 20 seconds, gradient annealing temperatures ranging from 49.7°C to 60.4°C for 30 seconds, and extension at 68°C for 1 minute, with a final extension at 68°C for 5 minutes. The production of the correct PCR
product was confirmed by electrophoresis on a 1% agarose gel in 1x TBE buffer at 100 V. The reactions containing the correct product size were combined and PCR purification was performed according to the QIAquick PCR Purification Kit and Handbook (QIAGEN). The PCR product was then digested using 10 U each of SalI and EcoRI in a buffer recommended by the enzyme suppliers. The 100 μl reaction was incubated at 37°C for 1.5 hours after which the DNA was purified using the QIAGEN protocol.

2.2.2.3 YY1 and RACK 1

The pOTB7:YY1 and pOTB7:RACK 1 plasmids were purified using the QIAprep Miniprep kit (QIAGEN) and linearized with BamHI and SmaI, respectively. PCR reactions (20 μl) containing approximately 4 ng of linearized DNA template were combined with 0.75 U of Platinum Pfx DNA polymerase (Invitrogen), 1x Pfx Amplification Buffer, 1mM MgSO₄, 0.3 μM of appropriate forward and reverse primers (Table 1), and a 0.3 mM mix of dNTPs. The reactions were then amplified in a Biometra T Personal thermal cycler with the following conditions: an initial 2 minute denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 68°C for 2 minutes, with a final extension of 2 minutes at 68°C. The production of the correct PCR product was confirmed by electrophoresis on a 1% agarose gel. The reactions containing the correct PCR product size were purified
<table>
<thead>
<tr>
<th>cDNA</th>
<th>Sequence of primer</th>
<th>Forward/Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciao 1</td>
<td>TGCCTCGGAATCCATGAAGGACTCGGTGGTGCA</td>
<td>04.68 forward</td>
</tr>
<tr>
<td>Ciao 1</td>
<td>CCAAGTGTGCACGTGTTATCATGAGGACGGCTTTTACGGCCG</td>
<td>04.69 reverse</td>
</tr>
<tr>
<td>RACK 1</td>
<td>CGCAGTGTCGACATGACTGAGCAGATGACCCTTTCGTG</td>
<td>05.18 forward</td>
</tr>
<tr>
<td>RACK 1</td>
<td>GCAATGCCGGGCGGCTAGCAGTGTGCCAATGGTC</td>
<td>05.19 reverse</td>
</tr>
<tr>
<td>YY1</td>
<td>CGTTCACCATGCGCTCAAGAACAATGCTTGGC</td>
<td>05.16 forward</td>
</tr>
<tr>
<td>YY1</td>
<td>CAGTCCGAAATCTTATCACTGGTTGTTTTGGCCTTAG</td>
<td>05.17 reverse</td>
</tr>
</tbody>
</table>

**Table 1.** Primers used in PCR to amplify the Ciao 1, RACK 1 and YY1 cDNAs encoded in pOTB7 plasmids in preparation for cloning into pGEX plasmids.
using a kit (QIAGEN) and restriction enzyme digests were performed in preparation for cloning. The RACK 1 PCR product was digested with 6 U each of Sall (NEB) and NotI (NEB) in a buffer recommended by the enzyme supplier. A similar reaction was carried out for the cDNA of YY1 using 6 U each of Ncol and EcoRI. The 35-40 μl reactions were incubated for 1.5 hours at 37°C and purified as outlined above.

2.2.3 Cloning of Protein Partners

The digested and purified Ciao 1, U2AF65, and RACK 1 cDNAs were cloned into compatible sites on the pGEX-4T-3 plasmid using the T4 DNA ligase system (Invitrogen). A partial YY1 cDNA encoding the zinc finger domain was cloned into the pET30a plasmid under the same conditions. The 40 μl ligation reactions were comprised of 2 units of T4 ligase (Invitrogen), 1x T4 ligase buffer, 20 ng of plasmid and 50 ng of DNA insert. The reactions were incubated at 16°C, overnight.

Each cloning reaction was then used to transform either the DH5α or TOP10 strains of E. coli (Invitrogen). An aliquot of each reaction (20 μl) was added to 100 μl of DH5α or TOP10 competent cells and incubated on ice for 5 minutes. The cells were then heat shocked at 42°C for 2 minutes and placed back on ice for 5 minutes. 800 μl of room temperature LB media was added and the cells were incubated at 37°C for 1 hour while shaking at 250 rpm. The cells were pelleted for 5 minutes at 6000 rpm using a Baxter Biofuge 13 table top centrifuge. The pellets were resuspended in 50 μl of LB media and plated on LB agar containing 50 μg/ml of the appropriate antibiotic and incubated at 37°C, overnight.
2.2.4 Screening of Cloning

2.2.4.1 Colony PCR (RACK 1, YY1)

Colony PCR was performed to ensure cloning was successful and the plasmids contained an insert of the correct size. Twenty individual colonies from each of the RACK 1 and YY1 plates were picked and used as templates for the colony PCR reactions. Each 10 μl reaction contained 1 unit of Taq DNA polymerase (Invitrogen), 1x Taq PCR buffer, 1.5 mM MgCl₂, 0.3 μM of appropriate forward and reverse primers (Table 2), 0.1 mM mix of dNTPs, and an individual colony template. The reactions were amplified with the following conditions; an initial denaturation at 94°C for 10 minutes, an initial annealing at 55°C for 30 seconds, and an initial extension at 72°C for 90 seconds, followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds and extension at 72°C for 90 seconds. The presence of the correct PCR product was confirmed by electrophoresis on a 1% agarose gel. Plasmids with putative inserts of the correct size were isolated from overnight 5 ml cultures seeded with their corresponding colonies and analyzed further by restriction enzyme digests.

2.2.4.2 Restriction Enzyme Digests (U2AF65, Ciao 1, RACK 1, YY1)

The cloning reactions were confirmed by restriction enzyme digests and DNA sequencing. Single colonies were picked at random and used to inoculate 5 ml of LB media containing 50 μg/ml of the correct antibiotic. Cultures were grown overnight at 37°C with shaking at 250 rpm. The plasmids from the overnight cultures were then purified using a QIAprep miniprep kit (QIAGEN). Approximately 200 ng of each
<table>
<thead>
<tr>
<th>Plasmid/cDNA of interest</th>
<th>Sequence of primer</th>
<th>Forward/reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-4T-3:RACK 1</td>
<td>GGGCTGGCAAGCCACGTTTGGT</td>
<td>04.60 forward</td>
</tr>
<tr>
<td>pGEX-4T-3:RACK 1</td>
<td>CCGGGAGCTGCATGTCAGAGG</td>
<td>04.61 reverse</td>
</tr>
<tr>
<td>pET30a:YY1</td>
<td>CGGCCGGTGTGAGCGGATAACCAATTC</td>
<td>04.46 forward</td>
</tr>
<tr>
<td>pET30a:YY1</td>
<td>GCCCGATGCTAGTTATGGCTGCAGG</td>
<td>04.47 reverse</td>
</tr>
</tbody>
</table>

Table 2. Primers used in colony PCR screening for successful cloning reactions of pGEX-4T-3:RACK 1 and pET30a:YY1.
purified plasmid was screened using the appropriate restriction enzymes (Table 3). The reactions were analyzed on 0.8-1% agarose gels and the pattern of restriction fragments were compared with the expected restriction fragment pattern. Plasmid isolates with the expected restriction fragment pattern were characterized further by DNA sequencing at the Centre for Biomedical Research DNA Sequencing Facility at the University of Victoria.

2.2.5 Overexpression and Purification of Recombinant Proteins

2.2.5.1 WT1 and Wx mutants, TFIIIA, ZAP1 and AZF1

WT1 (+KTS), the four Wx+ mutants and TFIIIAzf4-7 were kindly provided as purified proteins by Julie Foster as part of her Masters thesis. WT1 (-KTS) and the three Wx- mutants were kindly provided as purified proteins by Lindsay Bishop. Purified full length TFIIIA was kindly provided by Megumi Takiguchi. Purified *Tm*CBM4-2 was kindly provided by Alicia Lammerts van Bueren. ZAP1 and AZF1 were kindly provided as bacterial pellets by Dr. Romaniuk.

2.2.5.2 Recombinant GST Protein Expression and Purification

*Escherichia coli* BL21 competent cells were transformed (as previously described) with approximately 100 ng of pGEX plasmid. The cells were plated onto Luria Broth (LB) plates containing 50 µg/ml of ampicillin and were incubated for 18 hours at 37°C.
<table>
<thead>
<tr>
<th>Purified plasmid</th>
<th>Restriction enzymes for double digest</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX-4T-3:Ciao 1</td>
<td>EcoRI / SalI</td>
</tr>
<tr>
<td>pGEX-4T-3:U2AF65</td>
<td>BamHI / EcoRI</td>
</tr>
<tr>
<td>pGEX-4T-3:RACK 1</td>
<td>NorI / SalI</td>
</tr>
<tr>
<td>pET30a:YY1</td>
<td>NcoI / EcoRI</td>
</tr>
</tbody>
</table>

**Table 3.** Restriction enzymes used in double digest screening for successful cloning reactions of pGEX-4T-3:Ciao 1, pGEX-4T-3:U2AF65, pGEX-4T-3:RACK 1 and pET30a:YY1.
A single colony from each transformation was incubated in 5 ml of 2xYTA media (1.6% tryptone, 1% yeast extract, 0.5% NaCl, 100 μg/ml of ampicillin, pH 7.0) for 18 hours at 37°C, shaking at 250 rpm. A 200 ml volume of 2xYTA media was inoculated with 2 ml of the overnight culture of E. coli BL21 containing the pGEX expression plasmid. When the culture reached with an absorbance at 600 nm of at least 1.0, protein synthesis was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the temperature was lowered to 28°C. The cells were harvested 1-4 hours after induction by centrifugation at 5,000 rpm in a Beckman JA-16.250 rotor and the pellets were stored at -80°C.

The cell pellets were re-suspended in 10 ml of ice-cold 1xPBS binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 1mM PMSF, pH 7.3) and lysed by the french pressure cell (2 x each culture) or by ultrasonication (5x15 s pulses on ice with 1.5 min pauses between pulses). A solution of 20% Triton X-100 was added to a final concentration of 1% and the cell lysate was mixed gently by end-over-end rotation at room temperature for 30 minutes. The cell debris was then pelleted by centrifugation at 10,000 rpm in a Beckman JA-20 rotor for 10 minutes at 4°C.

The GST fusion proteins were then purified using a batch purification method. The crude protein solution was applied to 200 μl of a 50% slurry solution of Glutathione Sepharose 4B and rotated at room temperature for 30 minutes. The Glutathione Sepharose 4B matrix was washed three times with 1 ml of 1xPBS binding buffer and the proteins were eluted with 3x 100 μl of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The purified protein fractions were aliquoted and stored at -80°C. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay).
using bovine serum albumin (BSA) as a standard. The purity of individual protein preparations was assessed by 12.5-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2.5.3 Recombinant His Protein Expression and Purification

The expression and purification of recombinant His proteins were performed using a modified lab protocol. *E. coli* BL21 (DE3) star competent cells (Invitrogen) were transformed (as previously described) with approximately 30 ng of the His-tagged expression plasmid construct. The cells were plated onto LB plates containing 50 μg/ml of kanamycin and were incubated for 18 hours at 37°C. A single colony from each transformation was used to seed 5 ml of LB media containing 50 μg/ml of kanamycin that was incubated for 18 hours at 37°C with shaking at 250 rpm. A 250 ml volume of LB media containing 50 μg/ml of kanamycin was inoculated with 2.5 ml of the overnight culture of *E. coli* BL21 (DE3) containing the pET30a expression vector and incubated at 37°C with shaking. When the culture reached an absorbance of 1.0 at 595 nm, protein synthesis was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After an additional four hours incubation at 37°C with shaking, the cells were harvested by centrifugation at 5,000 rpm in a Beckman JA-16.250 rotor and stored at -80°C.

The cell pellets were resuspended in 10 ml of buffer A (10 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 250 mM NaCl, 10 mM PMSF, 10% glycerol, 5 mM DTT, 5 mM imidazole) and lysed using a french pressure cell (2 x each culture). The resulting cell lysates were then pelleted by centrifugation at 10,000 rpm in a Beckman JA-20 rotor and
the supernatants were discarded. The proteins were extracted from the inclusion bodies by resuspending in 5 ml of buffer B (buffer A containing 5 M urea) and gently mixed by end-over-end rotation at 4°C, overnight. The cell debris was pelleted again by centrifugation at 10,000 rpm at 4°C for 10 minutes.

The crude protein solution was then applied to a pre-equilibrated 1.5 ml HIS-select nickel affinity gel column (Sigma). The column was washed with 10 ml of buffer B, followed by a 2 ml wash of buffer B containing 50 mM imidazole. The proteins were eluted from the column with 1 ml of buffer B containing 150 mM imidazole and 1 ml of buffer B containing 250 mM imidazole. The protein concentrations were determined by the Bradford method using BSA as a standard. The purity and molecular weights of the individual protein preparations were assessed using 15% SDS-PAGE.

2.3 GST Pulldown Assays with Purified Proteins

A GST pulldown technique was employed to investigate the interaction between WT1 and its putative protein partners using recombinant GST and His-tagged fusion proteins. 5 μg of recombinant GST fusion protein was incubated in a final volume of 200 μl pulldown buffer (20 mM HEPES, 0.1% NP-40, 0.5 mg/ml BSA, 1mM DTT, and 20 μM ZnCl₂) and 20 μl of 50% slurry of Glutathione Sepharose 4B while gently rotating for 1 hour at room temperature. The Glutathione Sepharose 4B beads and bound proteins were then sedimented by centrifugation at 1600 rpm for 5 minutes and the beads were washed 3 times with 400 μl of pulldown buffer.

The His-tagged fusion protein (5μg) was pre-cleared in a final volume of 200 μl pulldown buffer and 10 μl of 50% Glutathione Sepharose 4B slurry while rotating at
room temperature for 20 minutes. The Glutathione Sepharose 4B matrix was sedimented by centrifugation at 1600 rpm and the supernatant was removed and added to the washed Glutathione Sepharose 4B matrix with the GST fusion protein bound. The two recombinant proteins were incubated together using gentle end-over-end rotation for 1 hour at room temperature. The reaction solution was then sedimented by centrifugation at 1600 rpm and the beads were washed three times with 400 μl of pulldown buffer. The pulldown reactions were then analyzed by SDS-PAGE and Western blotting.

2.3.1 Analysis of Pulldown Reactions

2.3.1.1 SDS-PAGE and Coomassie Staining

Pulldown reactions were analyzed using SDS-PAGE and coomassie staining. SDS-PAGE sample buffer (20 μl) was added to the sedimented beads from each pulldown reaction which were then heated for 5 minutes at 95°C. A 10 μl aliquot of each reaction was separated by 15% SDS-PAGE. In order to visualize proteins, gels were incubated for 60 minutes at room temperature in a Coomassie staining solution (0.2% w/v Brilliant Blue G (Sigma), 45% methanol, 45% water, 10% acetic acid) and then destained overnight in a solution containing 40% methanol, 10% acetic acid, and 40% water.

2.3.1.2 Western Blot

In some cases pulldown reactions were analyzed by Western blotting with monoclonal antibodies. After separation by 15% SDS-PAGE as described above, proteins in the gel were electrophoretically transferred to a nitrocellulose membrane at
100 V. The transfer was performed for 1 hour at 4°C in transfer buffer (22 mM Tris base, 192 mM glycine, and 20% v/v methanol, pH 8.1-8.4) using a Bio-Rad Mini Trans-Blot Cell. The blotted membranes were then incubated in 10 ml blocking solution (Invitrogen) on a rotary shaker for 30 minutes. The membrane was rinsed twice with 20 ml of distilled water and then incubated for 1 hour in 10 ml of the primary antibody, anti-His mouse monoclonal (Santa Cruz), which was diluted 1:2000 in blocking solution (Invitrogen). The membrane was then washed 3x 5 minutes in antibody wash (1xTBS; 10 mM Tris base, 137 mM NaCl, pH 7.6 and 0.1% Tween-20) and then incubated for 30 minutes in 10 ml of the secondary antibody, either goat anti-mouse IgG conjugated with alkaline phosphatase (Santa Cruz) or horse radish peroxidase (Abcam) diluted 1:2000 in blocking solution (Invitrogen). After the membrane was washed 3x 5 minutes with 20 ml of 1x TBS and 0.1% Tween-20 and rinsed 3x 2 minutes in 10 ml distilled water, it was placed on a piece of transparency plastic. For alkaline phosphatase antibodies, 1 ml of Bold APB Chemiluminescent Substrate (Molecular Probes) or CDP-Star AP-Substrate (Novagen) was applied to cover the entire surface of the membrane. After the reaction developed for 5 minutes, the membrane was lightly blotted to remove any excess reagent. Alternatively, for horse radish peroxidase antibodies, 2 ml of Luminol Reagent (Santa Cruz) was applied to cover the entire surface of the membrane and allowed to develop for 3 minutes. The membranes were exposed to x-ray film (BioMax MR film, Kodak) for 1-5 minutes.
2.4 Radioactive Proteins and GST Pulldown Assays

2.4.1 TnT PCR of pET30a:WT1(-KTS), pET30a:p43zf6-9, pET30a:WT1Δ4, pET30a:WT1Δ1

A set of TnT primers was designed which contained both a T7 RNA polymerase promoter and a eukaryotic translation initiation site (Kozak sequence) in the forward primer. The resulting PCR products contained the T7 RNA promoter and the Kozak sequence integrated into the 5' end permitting in vitro transcription and translation of the resulting RNA in a coupled transcription/translation extract (Promega). Each 20 μl PCR reaction consisted of 0.75 U Platinum Pfx DNA Polymerase (Invitrogen), 1x Pfx amplification buffer (Invitrogen), 0.3 mM dNTPs (dATP, dCTP, dGTP, dTTP), 1mM MgSO₄, 5 ng of DNA template, and 0.6 μM TnT forward primer (5’CGCTAATACGACTCACTATAGGGAGCCACCATGGACAGCCCCAGATCTG- 3’) and TnT reverse primer

(5’ATGCTAGTTATTGCTACGCGGTGACGCAGCCAACCTCAGCTTCCTTTCCG-3’). The reactions were amplified in a Biometra T Personal thermal cycler with an initial denaturation of 94°C for 2 minutes, and 30 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 68°C for 2 minutes, followed by a final extension at 68°C for 2 minutes. The production of the correct PCR product was confirmed by electrophoresis on 0.8-1% agarose gels. The reactions containing the correct PCR product size were purified using QIAquick columns (QIAGEN).
2.4.2 Gradient TnT PCR of pET30a:YY1

The amplification of YY1 cDNA was performed using a gradient PCR reaction. The PCR reaction and conditions were carried out as above, with the exception that annealing temperatures ranged between 50 and 60°C. The PCR product was verified through electrophoresis on a 1% agarose gel and purified as previously described.

2.4.3 Finger Swap Mutagenesis TnT PCR of WT1:YY1 Chimeric Sequences

Creation of the WT1:YY1 chimeric proteins was carried out with three 20 μl PCR reactions (Figure 7). The first two PCR reactions created the two templates required for the final PCR reaction. The first reaction used to create the chimeric protein, W12Y34, amplified the first two zinc finger sequences of WT1 in tandem with a region homologous to a portion of YY1 zinc finger 3 directly downstream of WT1 zinc finger 2. This template was entitled W12Y34-N. The second reaction amplified the sequence of YY1 zinc fingers 3 and 4 in tandem with a region homologous to a portion of WT1 zinc finger 2 directly upstream to YY1 zinc finger 3. The second template was entitled W12Y34-C. A similar set of PCR reactions were carried out to create the templates for the chimeric protein, Y12W34. These templates were provided as 1:50 diluted solutions by Dr. Romaniuk. Table 4 contains the primer sequences for the three-step PCR reactions.

The final PCR reaction to construct the W12Y34 chimeric sequence used both PCR products W12Y34-N and W12Y34-C as templates. The overlapping sequences in the two PCR products provided a full-length template for the final product. The resulting chimeric protein contained the first two zinc fingers of WT1 fused to zinc fingers three
Figure 7. Schematic of the three-step PCR reactions used to create the WT1:YY1 chimeric proteins. The top (PCR-1) and middle (PCR-2) panels represent the PCR reactions that create the W12Y34-N and W12Y34-C templates required for the third round of PCR. The lower panel depicts the final round of PCR and W12Y34, the final product. The Y12W34 chimeric protein was created in the same manner.
<table>
<thead>
<tr>
<th>PCR round</th>
<th>Sequence of primer</th>
<th>Forward/reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CGGCCGTTGTGAGCGGGATAACAATTCCC</td>
<td>forward</td>
</tr>
<tr>
<td>1</td>
<td>CGTGCACTGAAATGGTTTACACCTGTATGTCTC</td>
<td>reverse</td>
</tr>
<tr>
<td>2</td>
<td>GGAGAGAGACTCCTTCCAGTGATGAACCTTGTCAG</td>
<td>forward</td>
</tr>
<tr>
<td>2</td>
<td>GTAGCGGTCACGCTCGCGGTAACC</td>
<td>reverse</td>
</tr>
<tr>
<td>3</td>
<td>CGCTAATAACGACTCAGATAGGGAGCCACCAGTGACAGCCCAGATCTG</td>
<td>forward</td>
</tr>
<tr>
<td>3</td>
<td>ATGCTAGTTATTTGCTCAGCGGGTGAGCAGCAGCCAAGTCAGTTGCTCCTTTCGG</td>
<td>reverse</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PCR round</th>
<th>Sequence of primer</th>
<th>Forward/reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CGGCCGTTGTGAGCGGGATAACAATTCCC</td>
<td>forward</td>
</tr>
<tr>
<td>1</td>
<td>GTCCTACGGATGCCTGTGTGATGATAGGGAAC</td>
<td>reverse</td>
</tr>
<tr>
<td>2</td>
<td>GGAGAGAGACTCCTTCCAGTGATGAACCTTGTCAG</td>
<td>forward</td>
</tr>
<tr>
<td>2</td>
<td>GTAGCGGTCACGCTCGCGGTAACC</td>
<td>reverse</td>
</tr>
<tr>
<td>3</td>
<td>CGCTAATAACGACTCAGATAGGGAGCCACCAGTGACAGCCCAGATCTG</td>
<td>forward</td>
</tr>
<tr>
<td>3</td>
<td>ATGCTAGTTATTTGCTCAGCGGGTGAGCAGCAGCCAAGTCAGTTGCTCCTTTCGG</td>
<td>reverse</td>
</tr>
</tbody>
</table>

**Table 4.** Primers used in mutagenic TnT three-step PCR reactions for the creation of the WT1:YY1 chimeric proteins W12Y34 and Y12W34.
and four from YY1 (Figure 6). A similar set of reactions were carried out to create the chimeric protein, Y12W34. The Y12W34 protein contained the first two zinc fingers of YY1 fused to the third and fourth zinc fingers of WT1. Each 20 μl PCR reaction consisted of 0.75 U Platinum Pfx DNA polymerase (Invitrogen), 1x Pfx Amplification Buffer, 0.3 mM dNTP mix, 1mM MgSO4, 1 μl of each template, and 0.6 μM TnT PCR round 3 forward and reverse primers (Table 4). The reactions were amplified in a Biometra T personal thermal cycler with 5 cycles consisting of denaturation at 94°C for 2 minutes, annealing at 55°C for 30 seconds, and extension at 68°C for 1 minute, followed by 30 cycles of denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 68°C for 1 minute after which a final extension at 68°C for 3 minutes was carried out. The PCR products were verified to have the expected size by electrophoresis on 0.8% agarose gels and purified as described above. The chimeric PCR products were then sequenced to confirm that they contained the correct DNA sequences.

2.4.4 TnT T7 Quick Coupled Transcription/Translation Reactions

The TnT T7 Quick Coupled Transcription/Translation reaction uses a rabbit reticulocyte system which allows for the coupling of transcription from the phage polymerases with translation in eukaryotic extracts. The in vitro transcription/translation reactions consisted of 100 ng of TnT PCR product, 15 μCi of Redivue L-[^35]S] methionine (Amersham Biosciences), and 40 μl of TnT master mix (Promega). The 50 μl reactions were incubated for 90 minutes at 30°C and were then aliquoted and stored at -80°C.

The efficiency of the reactions was analyzed by SDS-PAGE, autoradiography, and exposure to a phosphorimaging screen. 1 μl of each reaction was characterized by
15% SDS-PAGE. The gel was fixed for 30 minutes in 50% methanol, 10% acetic acid and then soaked in 7% methanol, 7% acetic acid and 10% glycerol for 5 minutes. The gels were dried for 1 hour at 80°C on a slab drier and exposed to x-ray film (Kodak) for 1-3 days and to a Molecular Dynamics phosphor screen for 1-5 days and visualized using the STORM imaging system.

2.4.5 GST Pulldowns with $^{35}$S-labelled Proteins

The interaction of possible protein partners was further investigated using a GST pulldown technique involving $^{35}$S-labelled proteins. GST fusion proteins were purified as previously described in section 2.2.5.2 and 5 µg fractions were diluted in 200 µl of binding buffer (25 mM HEPES pH 7.5, 12.5 mM MgCl$_2$, 0.1% NP-40, 150 mM KCl, 1 mM DTT, 150 µg/ml BSA). The solution was mixed by gentle end-over-end rotation for 10 minutes at room temperature with 20 µl of 50% Glutathione Sepharose 4B slurry. Similar “counts” of $^{35}$S-labelled proteins were added to the solution and incubated for an additional hour while gently rotating. The reaction solution was sedimented by centrifugation at 1600 rpm for 5 minutes and the Glutathione Sepharose 4B matrix was washed with 5x 1 ml of wash buffer (20 mM Tris base pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40).

The reactions were separated on 15% SDS-PAGE as described in section 2.3.1.1 and analyzed first by exposure to x-ray film for 1 to 5 days. For semi-quantitative analysis gels were exposed to a Molecular Dynamics phosphor screen for 1-5 days and then visualized using the STORM imaging system.
Chapter 3. Results

3.1 Introduction and Overview

A series of GST pulldown assays were employed to explore the mechanism in which WT1 interacts with its protein partners. To determine which zinc finger(s) are critical in protein-protein interactions several sets of mutant proteins were produced. The initial set of finger swap mutants were previously created through site directed mutagenic PCR. These four Wx+ mutants each had a single α helical region of WT1 replaced with the corresponding α helical region from TFIIIAzf4-7. These mutants were created based on a series of previous findings from x-ray crystallography, mutagenic studies and quantitative binding assays. Several groups have crystallized complexes containing DNA-zinc finger protein complexes and RNA-zinc finger protein complexes (Pavletich and Pabo, 1991; Nolte et al., 1998; Lu et al., 2003). From these structures it can be seen that the α helical regions of the zinc finger proteins are directly responsible for nucleic acid-protein interactions. Furthermore, our lab has performed extensive studies on the nucleic acid ligands of TFIIIA and WT1. One study involved a series of finger swap mutants in which TFIIIA had a single α helical region replaced with the corresponding α helical region from WT1. From this study it was found that certain mutants abolished the DNA and RNA binding abilities of TFIIIA (Hamilton et al., 2001). The GST pulldown assays of these mutants were performed using purified recombinant proteins. The second set of mutant proteins consisted of chimers of WT1 and YY1. The W12Y34 chimeric protein contained the first two zinc fingers of WT1 fused to the third and fourth zinc fingers of YY1. The Y12W34 chimeric protein contained the first two zinc fingers of YY1 fused to the third and fourth zinc fingers of WT1. These two chimeric proteins
were created based on previous studies that the WT1 and YY1 proteins had separate and distinct protein ligands (Johnstone et al., 1996; Johnstone et al., 1998). The effects of single finger deletion mutants were also analyzed using the N-terminal truncation mutant, WT1Δ1, and C-terminal truncation mutant, WT1Δ4. These GST pulldown assays were performed using radiolabelled *in vitro* transcribed and translated proteins.

### 3.2 Diagnostic Screening of Cloning

#### 3.2.1 Diagnostic Colony PCR

The RACK1 and YY1 cDNA sequences were ligated into the pGEX-4T-3 and pET30a expression plasmids, respectively. The cloning reactions were used to transform *E. coli* DH5α or TOP10 (Invitrogen) competent cells and were plated onto antibiotic containing LB plates to obtain single colonies. Colony PCR was performed to ensure the ligation of the cDNA insert and the expression vector had occurred and the colonies did not result from re-ligation of an empty plasmid. Figure 8 is representative of a typical colony PCR reaction. Plasmids pET30a containing the YY1 cDNA appear in lanes 1 and 3 through 10 with an apparent product size of 750 bp. Lane 2 is representative of a negative reaction, in which re-ligation of the empty plasmid occurred. Similar reactions were carried out for the identification of pGEX-4T-3:RACK1 plasmids. Plasmids that contained successful ligation reactions yielded PCR products with the expected 980 bp size. Plasmids from colonies that produced PCR products with the correct banding pattern were then purified and screened further by restriction enzyme digests.
Figure 8. Identification of pET30a:YY1 plasmids by colony PCR. Lanes 1 and 3 through 10 show plasmids which contain the putative YY1 sequence. Lane 2 shows a negative reaction possibly resulting from re-ligation of an empty plasmid. The products were electrophoresed on a 1% agarose gel in TBE buffer at 100 V and visualized by ethidium bromide staining.
3.2.2 Diagnostic Restriction Enzyme Digests

The Ciao 1 and U2AF65 cDNA sequences were ligated into the pGEX-4T-3 plasmids. The cloning reactions were used to transform *E. coli* DH5α competent cells. To ensure successful cloning, several colonies were chosen at random for plasmid purification. To confirm their identities the putative purified plasmids pGEX-4T-3:Ciao 1, pGEX-4T-3:U2AF65, pGEX-4T-3:RACK1 and PET30a:YY1 were analyzed using restriction enzyme digests. Figure 9 illustrates the restriction digest of the putative pGEX-4T-3:U2AF65 plasmid using *BamHI* and *EcoRI*. Successful cloning can be observed with the restriction fragment pattern of 1450 bp and 4900 bp, representing the cDNA insert and plasmid respectively. Successful reactions can be seen in lanes 2, 4, 6, and 10, while lane 8 contains a negative reaction resulting from possible contamination. The successful cloning of pGEX-4T-3:Ciao 1 was determined by digestion with *EcoRI* and *SalI* and yielded the expected fragment pattern of 1052 bp for the Ciao 1 cDNA insert. The digestion of pGEX-4T-3:RACK1 using *NotI* and *SalI* produced the expected fragment pattern of 980 bp for the RACK1 cDNA insert. The identity of plasmid PET30a:YY1 was confirmed by digestion using *NcoI* and *EcoRI* and produced the expected fragment pattern of 400 bp for the YY1 cDNA insert.

Plasmid isolates with the expected restriction fragment patterns were then sequenced at the CBR Sequencing Facility at UVic. Clones that were verified to contain the correct DNA sequence were subsequently used for protein expression and purification.
Figure 9. Identification of pGEX-4T-3:U2AF65 plasmids by restriction enzyme digests. Lanes 1, 3, 5, 7 and 9 show undigested putative pGEX-4T-3:U2AF65 plasmids. Lanes 2, 4, 6, 8 and 10 show the corresponding plasmids digested with BamHI and EcoRI. Successful reactions can be seen in lanes 2, 4, 6 and 10 with the correct banding pattern of 1450 and 4900 bp. Lane 8 shows a negative reaction from possible contamination. The products were electrophoresed on a 1% agarose gel in TBE buffer at 100 V and visualized by staining with ethidium bromide.
3.3 Overexpression and Purification of Recombinant Proteins

3.3.1 GST Recombinant Proteins

The pGEX plasmids were used to transform BL21, an expression strain of *E. coli*. BL21 is a protease-deficient cell line which allows for the utmost expression of full-length GST recombinant proteins. The pGEX vectors are under the control of the tac promoter and can be induced by IPTG, a lactose analog, which alleviates repression from the *lacI* gene product. The addition of IPTG allows for the expression of high levels of recombinant GST protein.

The GST fusion proteins were purified from bacterial lysates using affinity chromatography on Glutathione Sepharose 4B and eluted from the affinity medium under non-denaturing conditions. The molecular weights and purity of the proteins were verified through electrophoresis on 15% SDS-PAGE and coomassie staining. The purified fractions of GST-Ciao 1 are illustrated in Figure 10. GST-Ciao 1 migrates with an approximate apparent molecular weight of 65 kDa. The predicted and approximate apparent molecular weights of GST, GST-Ciao 1, GST-U2AF65, GST-par4, GST-p53 and GST-RACK1 are presented in Table 5. The concentrations of the proteins were determined by a Bradford protein assay using BSA as a standard. The concentrations of the purified proteins were in the range of 0.2-12 µg/µl (Table 5).

3.3.2 His Recombinant Proteins

The pET100 and pET30a plasmids were used to transform *E. coli* BL21 (DE3) and BL21 (DE3) pLysS cells. The BL21 (DE3) strain is a λDE3 lysogen and carries a
Figure 10. SDS-PAGE of GST-Ciao 1 protein fractions purified by a batch purification method. Lanes 1-3 contain 10 μl aliquots of each sample. The proteins were separated on a 15% acrylamide gel at 200 V for 1 hour and visualized by coomassie staining.
<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>Predicted molecular weight (kDa)</th>
<th>Approximate apparent molecular weight (kDa)</th>
<th>Average fraction concentration (μg/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST</td>
<td>26</td>
<td>30</td>
<td>6.60</td>
</tr>
<tr>
<td>GST-Ciao 1</td>
<td>64.4</td>
<td>65</td>
<td>4.17</td>
</tr>
<tr>
<td>GST-U2AF65</td>
<td>91</td>
<td>91</td>
<td>0.43</td>
</tr>
<tr>
<td>GST-par4</td>
<td>62</td>
<td>80</td>
<td>0.46</td>
</tr>
<tr>
<td>GST-p53</td>
<td>70</td>
<td>70</td>
<td>0.26</td>
</tr>
<tr>
<td>GST-RACK1</td>
<td>64</td>
<td>60</td>
<td>0.80</td>
</tr>
<tr>
<td>His-YY1</td>
<td>21</td>
<td>25</td>
<td>4.23</td>
</tr>
<tr>
<td>His-AZF1</td>
<td>19</td>
<td>25</td>
<td>2.20</td>
</tr>
<tr>
<td>His-ZAP1</td>
<td>18</td>
<td>25</td>
<td>2.85</td>
</tr>
<tr>
<td>His-p43zf1-4</td>
<td>20</td>
<td>30</td>
<td>0.24</td>
</tr>
<tr>
<td>His-p43zf6-9</td>
<td>18.5</td>
<td>25</td>
<td>0.30</td>
</tr>
</tbody>
</table>

**Table 5.** The predicted and apparent molecular weights and fraction concentration of the recombinant proteins GST, GST-Ciao 1, GST-par4, GST-p53, GST-U2AF65, His-YY1, His-ZAP1, His-p43zf1-4 and His-p43zf6-9.
copy of the T7 RNA polymerase gene which is controlled by the \textit{lacUV5} promoter. The pET expression vectors are under the control of the T7 promoter. High levels of protein expression can be induced through the addition of IPTG which displaces the \textit{lacI} protein from the \textit{lac} operator present in both the plasmid and host cell. The alleviation of repression allows for the production of T7 RNA polymerase from the host cell which transcribes the gene of interest in the pET plasmid.

The His fusion proteins were purified from bacterial lysates using affinity chromatography on a HIS-select nickel column and eluted under denaturing conditions. The purity and molecular weights of the proteins were confirmed by 15\% SDS-PAGE and coomassie staining. The predicted and approximate apparent molecular weights of YY1, AZF1, ZAP1, the C-terminal truncation mutant p43zf1-4, and the N-terminal truncation mutant p43zf6-9 are presented in Table 5. The concentrations of the purified proteins were determined by a Bradford protein assay using BSA as a standard and were in the range of 0.25-10 \mu g/\mu l (Table 5).

### 3.4 Purified Recombinant Protein GST Pulldown Method

#### 3.4.1 WT1 (+KTS) and Four Wx+ Site-directed Mutants

The mechanism of interaction between WT1 and its protein partners was probed through several variations of a GST pulldown assay. The initial set of pulldown reactions involved the use of purified, bacterially-expressed, recombinant proteins. The reactions consisted of GST fusion proteins and recombinant His WT1 (+KTS) wild type and the four Wx+ mutant proteins. The Wx+ mutants were created by substituting a single
α helical region of WT1 (+KTS) zinc fingers 1-4 with a single α helical region from the corresponding zinc fingers 4-7 of TFIIIA. The Wx+ mutants were expected to visibly reduce the protein binding ability of WT1 revealing the zinc finger(s) critical for binding. In order to determine if the mutations made within the α helical regions of WT1 (+KTS) resulted in a decrease in protein binding ability, a qualitative approach was used. Analysis of the relative band intensities of the Wx+ mutant reactions were compared with the WT1 (+KTS) wild type reactions. Figure 11 illustrates the interaction between GST-p53 and WT1 (+KTS) wild type and the four Wx+ mutant proteins. The GST portion alone was assayed to ensure the interactions were not a result of the recombinant portion of the GST proteins. Analysis of the western blot reveals the Wx+ mutants have no significant effect on the binding ability to GST-p53 in relation to wild type WT1 (+KTS). Similar reactions were carried out under the same experimental conditions for the GST fusion proteins par4 and Ciao 1 and yielded equivalent results.

3.4.2 WT1 (-KTS) and Three Wx- Site-directed Mutants

WT1 has the ability to bind to DNA and RNA with high specificity. The binding affinity of WT1 with nucleic acids has been found to be isoform-dependent. To ensure the interactions observed between the GST fusion proteins and the Wx+ mutants were not isoform specific, the GST pulldown reactions were repeated using WT1 (-KTS). A set of previously created mutant proteins of the WT1 (-KTS) protein were utilized. The three Wx- mutants were created in a similar manner as the Wx+ mutants, consisting of single α helical swaps from TFIIIAzf4-7. Figure 12 illustrates WT1 (-KTS) wild type and the three Wx- mutant proteins probed against the GST fusion proteins Ciao 1, par4, p53 and
Figure 11. Western blot of GST pulldown reactions between GST-p53 and WT1 (+KTS) wild type and Wx+ mutants. Each reaction was separated on a 15% acrylamide gel for 1 hour at 200 V and then electrophoretically transferred to a nitrocellulose membrane for 1 hour at 100 V. The primary antibody used was a mouse monoclonal against the His epitope and was diluted 1:2000. The blot was developed with chemiluminescent substrate and exposed to X-ray film for 4 minutes.
Figure 12. SDS-PAGE of GST pulldown reactions of WT1 (-KTS) wild type and Wx-mutants probed against GST fusion proteins Ciao 1, p53 and par4. 10 ul of each reaction was separated on 15% SDS-PAGE and visualized by coomassie staining. Panel A: WT1 (-KTS) wild type, Panel B: Wx- mutant 1b, Panel C: Wx- mutant 2b, Panel D: Wx-mutant 3b, NEB: New England Biolabs pre-stained protein marker.
GST alone. To determine if the $\alpha$ helical mutations resulted in a decrease in protein binding ability, the band intensities from the Wx- mutants reactions were compared to wild type WT1 (-KTS) reactions. Again, the Wx- mutants did not produce any visible reduction in protein binding affinity in comparison to wild type WT1 (-KTS).

Furthermore, to assess any differences in binding ability between the WT1 isoforms, the reaction intensities of WT1 (-KTS) and WT1 (+KTS) were compared. From this it was determined that the protein binding ability of the WT1 protein is not significantly affected by the presence or absence of the KTS splice insert.

3.4.3 Rationale for Further Analysis

The resulting interactions between the Wx mutants and the GST protein partners were unexpected. It was anticipated that one or more $\alpha$ helical swaps would result in a visibly reduced protein-protein interaction. Due to these unexpected findings, further analysis was required to probe the manner of WT1 protein binding.

In order to continue analyzing the protein interactions of WT1 it was vital to develop a clear understanding why the Wx mutants did not reduce protein binding. There were several possible explanations why the finger swap mutants did not disrupt WT1 protein-protein interactions. First, the protein-interaction site of WT1 may not be located within the $\alpha$ helical region of the zinc fingers. Second, the interaction may be specific for zinc finger proteins but non-specific for WT1. Third, the interaction may involve more than one zinc finger of WT1.
3.4.4 Screening for Potential Zinc Finger Donors

A series of GST pulldown reactions were carried out to determine an explanation for the unanticipated interactions of the Wx mutants. The first pulldown reaction investigated the possible interaction between GST-Ciao 1 and the zinc finger donor peptide TFIIIAzf4-7. Figure 13 illustrates a western blot of a GST pulldown assay between GST-Ciao 1 and TFIIIAzf4-7. Indeed, it can be seen that an interaction occurs between TFIIIAzf4-7 and GST-Ciao 1 but not with GST alone. This interaction clarifies the previous findings of the Wx mutants and GST-Ciao 1 since TFIIIAzf4-7 is not a suitable zinc finger donor.

To identify a potential zinc finger donor, several four-zinc finger-containing proteins were assayed in pulldown reactions with Ciao 1. Two zinc finger peptides of p43 were analyzed in GST pulldown reactions with GST-Ciao 1. From Figure 14 it can be seen that there is an interaction between GST-Ciao 1 and both p43zf1-4 and p43zf6-9 proteins. Similar pulldown reactions were carried out with the four-zinc finger proteins YY1, ZAP1, and AZF1. Again, it was seen that all three zinc finger proteins are present in the GST-Ciao 1 reactions whereas no interaction can be detected between the zinc finger proteins and GST alone (results not shown).

Further analysis was performed to ensure the protein-protein interactions were occurring as a result of the zinc finger protein and not the recombinant His portion. Figure 15 depicts the interaction of GST-Ciao 1 and a non-His tag version of TFIIIAzf4-7. From this figure it can be seen that an interaction occurs between TFIIIAzf4-7 and GST-Ciao 1 but no interaction can be detected between TFIIIAzf4-7 and GST alone. Furthermore, a pulldown assay involving GST-Ciao 1 and TmCBM4-2 was performed.
Figure 13. Western blot of GST pulldown reactions between TFIIIAzf4-7 and GST-Ciao 1. Lanes 1 and 2 show pulldown reactions of GST-Ciao 1 and TFIIIAzf4-7. Lanes 2 and 4 show pulldown reactions of GST and TFIIIAzf4-7. Lane 5 shows the 20% reaction input (1μg) of TFIIIAzf4-7. Each reaction was separated on 15% SDS-PAGE for 1 hour at 200 V and then electrophoretically transferred to a nitrocellulose membrane for 1 hour at 100 V. The primary antibody used was a 1:2000 dilution of mouse monoclonal against the His epitope. The blot was developed with chemiluminescent substrate and exposed to x-ray film for 6 minutes.
Figure 14. Western blot of GST pulldown reactions between p43zf1-4, p43zf6-9 and GST-Ciao 1. Lanes 1-3 show reactions with p43zf1-4. Lanes 4-6 show reactions with p43zf6-9. 10 μl of each reaction was separated on a 15% acrylamide gel for 1 hour at 200 V and then electrophoretically transferred to a nitrocellulose membrane for 1 hour at 100 V. The primary antibody used was a mouse monoclonal against the His epitope and was diluted 1:2000. The blot was developed with chemiluminescent substrate and exposed to x-ray film for 6 minutes.
Figure 15. SDS-PAGE of GST pulldown reactions between TFIIIAzf4-7 and GST-Ciao 1. Lane 1: 20% input (1 µg) TFIIIAzf4-7, lanes 2 and 4: TFIIIAzf4-7 and GST-Ciao 1 reactions, lanes 3 and 5: TFIIIAzf4-7 and GST reactions. 10 µl of each reaction was separated by 15% SDS-PAGE for 1 hour at 100 V and visualized by coomassie staining.
TmCBM4-2 is a recombinant His protein which does not contain any zinc finger motifs. Again, it was seen that no visible interaction between the two proteins. Therefore, from this data it can be assumed that the interaction is not due to the recombinant portion of the proteins but can be attributed to the zinc finger properties of the proteins.

3.5 Radioactive Proteins and GST Pulldown Assays

To continue characterizing the protein-interactions of WT1, a GST pulldown method using \textit{in vitro} translated proteins was employed. There are several possible explanations to account for why the initial pulldown reactions were yielding non-specific interactions between all the zinc finger proteins and the WT1 protein ligands. First, purification of the His fusion proteins was performed under denaturing conditions and proper re-folding may not occur. Second, the pulldown conditions contained only the proteins of interest, whereas in a physiological context a vast number of other proteins would be present. Third, the protein concentrations used in the assays far exceed the levels found in a cellular context. The use of a eukaryotic system to express the zinc finger proteins may alleviate some of these possible problems that may be interfering with the results of the pulldown assays.

3.5.1 TnT PCR Reactions

The TnT PCR reactions incorporate both a eukaryotic translation start site and a T7 RNA polymerase promoter site into the cDNA sequence. The creation of these TnT PCR products allows for the coupled \textit{in vitro} transcription and translation of the zinc
Figure 16. Product of TnT PCR amplification of WT1 (-KTS). 2 μl of PCR product was electrophoresed on a 1% agarose gel in TBE buffer at 100 V and was visualized with ethidium bromide staining. Lane 1: 1 kb ladder, lane 2: quantitative ladder, lane 3: WT1 (-KTS) PCR product.
<table>
<thead>
<tr>
<th>cDNA</th>
<th>Predicted size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1 (-KTS)</td>
<td>650</td>
</tr>
<tr>
<td>YY1</td>
<td>581</td>
</tr>
<tr>
<td>W12Y34</td>
<td>644</td>
</tr>
<tr>
<td>Y12W34</td>
<td>587</td>
</tr>
<tr>
<td>WT1Δ1</td>
<td>503</td>
</tr>
<tr>
<td>WT1Δ4</td>
<td>539</td>
</tr>
<tr>
<td>p43zf6-9</td>
<td>564</td>
</tr>
</tbody>
</table>

Table 6. Predicted sizes of TnT PCR products for WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and p43zf6-9.
finger proteins. Figure 16 is representative of a typical TnT PCR reaction depicting the 400 bp product of WT1 (-KTS). Similar TnT PCR reactions were carried out for YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4, and p43zf6-9 which were analyzed by electrophoresis on agarose gels. Table 6 represents the predicted band sizes of the TnT PCR products.

The TnT PCR products with the expected banding patterns were purified and then sequenced at the CBR Sequencing Facility at UVic. The PCR products that were verified to contain the correct DNA sequence were subsequently used for in vitro protein expression.

3.5.2 In vitro Transcription and Translation of $^{35}$S-labeled Proteins

The rabbit reticulocyte system present in the TnT T7 Quick Coupled reactions enabled the coupling of transcription from the phage polymerases with translation in eukaryotic extracts. Post-translational analysis of the $^{35}$S-labeled proteins was performed by electrophoresis on 15% SDS-PAGE and autoradiography. Figure 17 is an autoradiograph depicting 1 μl aliquots of the WT1 (-KTS) and YY1 TnT reactions yielding the approximate apparent molecular weights of 25 and 16.5 kDa respectively. Similar reactions were carried out for the TnT proteins WT1Δ1, WT1Δ4, W12Y34, Y12W34, and p43zf6-9. The predicted and approximate apparent molecular weights of the TnT proteins are presented in Table 7.
Figure 17. Autoradiogram of *in vitro* transcription and translation reactions of WT1 (-KTS) and YY1. 1 μl of each reaction was separated on a 15% acrylamide gel for 1 hour at 200 V and exposed to x-ray film for 18 hours. Lane 1: WT1 (-KTS), lane 2: YY1.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Approximate apparent molecular weight (kDa)</th>
<th>Predicted molecular weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1 (-KTS)</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>YY1</td>
<td>16.5</td>
<td>15.6</td>
</tr>
<tr>
<td>W12Y34</td>
<td>22</td>
<td>16.9</td>
</tr>
<tr>
<td>Y12W34</td>
<td>20</td>
<td>16.2</td>
</tr>
<tr>
<td>WT1Δ1</td>
<td>16.5</td>
<td>13.5</td>
</tr>
<tr>
<td>WT1Δ4</td>
<td>16.5</td>
<td>14.5</td>
</tr>
<tr>
<td>p43zf6-9</td>
<td>20</td>
<td>15.2</td>
</tr>
</tbody>
</table>

**Table 7.** The approximate apparent and predicted molecular weights for the *in vitro* translated proteins WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and p43zf6-9.
3.5.3 Pulldowns with $^{35}$S-labelled Proteins

3.5.3.1 Screening for Potential Zinc Finger Donors

The investigation of WT1 protein-protein interactions was furthered with the use of $^{35}$S-radiolabelled proteins in GST pulldown assays. The initial series of pulldown reactions were performed to isolate a potential zinc finger donor to create a set of chimeric mutant proteins. The chimeric proteins should allow the determination of which zinc finger(s) are involved in protein-protein interactions of WT1 by a visual reduction in protein binding.

To determine a potential zinc finger donor, reactions involving GST-Ciao 1 and several four-zinc finger-containing proteins were performed. Figure 18 depicts a series of GST pulldown assays involving the zinc finger proteins WT1 (-KTS), YY1 and p43zf6-9 and the GST fusion proteins Ciao 1, RACK1 and GST alone. From this figure a significant interaction between GST-Ciao 1 and WT1 (-KTS) can be observed, while there is no apparent interaction between GST alone and WT1 (-KTS). A significant interaction between GST-Ciao 1 and p43zf6-9 can also be observed. The autoradiogram displays approximately equal band intensities for both the WT1 (-KTS) and p43zf6-9 reactions, excluding p43zf6-9 as a potential zinc finger donor. A negligible interaction can be visualized between YY1 and all three GST fusion proteins making YY1 a suitable zinc finger donor.
Figure 18. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, p43zf6-9 and GST fusion proteins Ciao 1, RACK1 and GST. Lanes 1-4: WT1 (-KTS) reactions, lanes 5-8: YY1 reactions, lanes 10-12: p43zf6-9 reactions. 10 µl of each reaction was separated on a 15% acrylamide gel for 1 hour at 200 V and exposed to x-ray film for 56 hours.
3.5.3.2 Pulldowns with WT1 Deletion Mutants and Chimeric Proteins

The initial series of pulldown reactions revealed the zinc finger protein YY1 does not interact with GST-Ciao 1 providing YY1 as a suitable zinc finger donor. The interaction of WT1 and its protein partners was probed further using chimeric WT1:YY1 proteins and single-finger deletion mutants of WT1 (-KTS). Three individual trials were conducted to observe the binding of the GST fusion proteins Ciao 1, par4 and U2AF65 with the zinc finger proteins YY1, WT1 (-KTS), the two deletion mutants WT1Δ1 and WT1Δ4, and the chimeric proteins W12Y34 and Y12W34.

Figure 19 illustrates a typical GST pulldown assay involving GST-Ciao 1 and WT1 (-KTS), YY1, WT1Δ1, WT1Δ4, W12Y34 and Y12W34. From this figure a significant interaction between WT1 (-KTS) and GST-Ciao 1 can be observed, while no apparent interaction occurs between GST-Ciao 1 and YY1. The interactions between GST-Ciao 1 and the deletion mutants WT1Δ1 and WT1Δ4 both had reduced binding abilities for GST-Ciao 1 in relation to WT1 (-KTS) wild type. The deletion of WT1 zinc finger 4 appears to disrupt the interaction with GST-Ciao 1 to a slightly lesser degree than the deletion of zinc finger 1. It can also be seen that the chimeric proteins W12Y34 and Y12W34 have reduced affinities for GST-Ciao 1 in respect to WT1 (-KTS) wild type. Although the chimers bind to a much lesser degree than the WT1 (-KTS) wild type protein, both chimers have similar affinities for GST-Ciao 1.

A similar set of GST pulldown reactions were carried out for GST-U2AF65 and WT1 (-KTS), YY1, WT1Δ1, WT1Δ4, W12Y34 and Y12W34. Figure 20 depicts a typical GST pulldown assay for GST-U2AF65. The pattern of binding between GST-
Figure 19. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and GST-Ciao 1. 10 μl of each reaction was separated on 15% acrylamide gels for 1 hour at 200 V and exposed to x-ray film for 56 hours.
Figure 20. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and GST-U2AF65. 10 μl of each reaction was separated on 15% acrylamide gels for 1 hour at 200 V and exposed to x-ray film for 56 hours.
U2AF65 and the zinc finger proteins appears to be analogous to that found with GST-Ciao 1.

Using the same reaction conditions, three individual trials of GST pulldown reactions were also carried out for GST-par4. Figure 21 illustrates an individual GST pulldown trial for GST-par4 and the six zinc finger proteins. Although the binding results appear similar to the previous findings of Ciao 1 and U2AF65, there is one notable exception. The W12Y34 chimeric protein displays a distinct decrease in binding with par4 in comparison to the Y12W34 chimeric protein.
Figure 21. Autoradiogram of GST pulldown reactions between WT1 (-KTS), YY1, W12Y34, Y12W34, WT1Δ1, WT1Δ4 and GST-par4. 10 μl of each reaction was separated on 15% acrylamide gels for 1 hour at 200 V and exposed to x-ray film for 56 hours.
Chapter 4. Discussion

Zinc finger proteins comprise a large family of multifunctional proteins. The classical C$_2$H$_2$ zinc finger protein makes up approximately 1% of all mammalian proteins and is found in other eukaryotes as well as prokaryotes (Iuchi, 2001). The zinc finger is a small functional motif which acts as an interaction module allowing it to associate with molecules such as DNA, RNA and proteins (Laity et al., 2001). These interactions implicate zinc finger proteins in a diverse range of cellular processes including replication, repair and transcription (Krishna et al., 2003). Over the past several decades, numerous studies have provided a general framework for the interactions of zinc finger proteins and nucleic acids. More recently, studies have been focusing on characterizing zinc finger protein-protein interactions. Further analysis will provide a better understanding of the full realm of functions of these complex proteins.

The present study focuses on the zinc finger protein WT1. WT1 is a tumor suppressor protein involved in controlling development and normal physiology of the genitourinary tract (Pritchard-Jones et al., 1990). The WT1 gene product has multiple roles in development and disease; mutations in WT1 result in several syndromes afflicting the genitourinary tract (Orkin et al., 1984; van Heyningen et al., 1990; Coppes et al., 1993; Little et al., 1993; Wagner et al., 2003). Originally discovered as a transcription factor (Call et al., 1990), WT1 has also been shown to display specific interactions with RNA and proteins. The WT1 protein has four main isoforms resulting from two alternative splicing events of the primary transcript. The first alternative splice site controls inclusion or exclusion of exon 5 which encodes an additional 17 amino acids
between the N-terminal regulatory domain and the C-terminal zinc finger domain (Haber et al., 1991). The second alternative splicing event occurs at the end of exon 9 and results in the insertion of three amino acids (KTS) between zinc fingers 3 and 4 (Haber et al., 1991). The subnuclear localization pattern of the two isoforms suggests they are involved in distinct cellular functions. The WT1 (-KTS) isoform is found localized diffusely throughout the nucleoplasm (Englert et al., 1995) and functions primarily as a transcription factor. Several putative gene targets of WT1 (-KTS) have been identified and include bcl-2 (Hewitt et al., 1995; Mayo et al., 1999), c-myc (Hewitt et al., 1995; Han et al., 2004), and amphiregulin (Lee et al., 1999). The WT1 (+KTS) isoform has been thought to play a role in posttranscriptional processes due to its localization in nuclear speckles (Larsson et al., 1995). Furthermore, WT1 (+KTS) has been shown to associate with the splicing protein, U2AF65, and is able to directly incorporate into spliceosomes (Davies et al., 1998). Although the WT1 (+KTS) isoform has been shown to bind RNA with high affinity (Caricasole et al., 1996), a biological target has yet to be identified. Despite differences in nucleic acid affinity between the two isoforms, both WT1 (+KTS) and (-KTS) interact with proteins. Studies have identified a diverse group of WT1 protein partners that include p53 (Maheswaran et al., 1993; Scharnhorst et al., 2000), par4 (Johnstone et al., 1996; Richard et al., 2001), Ciao 1 (Johnstone et al., 1998), and U2AF65 (Davies et al., 1998). Assays using WT1 C-terminal deletion mutants have demonstrated that protein-protein interactions rely on the zinc finger domain of WT1, although the exact mechanism of binding has not yet been elucidated.

Previous work in our lab has concentrated on the nucleic acid binding abilities of zinc finger proteins such as TFIIBA and WT1. Extensive studies using quantitative
binding assays have examined the interactions between the \( \alpha \) helices of the zinc finger domain and nucleic acids. Using site-directed mutagenesis the \( \alpha \) helical regions have been shown to be critical for nucleic acid binding (Borel et al., 1996; Hamilton et al., 2001; Zhai et al., 2001). The purpose of the present study was to further characterize the specific zinc finger(s) involved in WT1-protein interactions and potentially identify amino acids critical for protein binding. It was hypothesized that specific amino acid residues within the \( \alpha \) helices contribute to protein-protein interactions. The use of site-directed mutagenesis within the zinc finger regions could identify which fingers are important for binding.

4.1 Wx Mutants of WT1 and TFIIIAzf4-7

To explore the manner of WT1-protein binding a set of WT1 mutants consisting of the C-terminal zinc finger region were created using site-directed mutagenesis. The creation of the Wx+ mutants was based on previous studies performed in our lab exploring the interaction between TFIIIA and its nucleic acid ligands. Each mutant had a single \( \alpha \) helical region of TFIIIAzf4-7 replaced with a single \( \alpha \) helical region from WT1zf1-4. The study demonstrated that specific zinc finger replacements had the ability to abolish both the specific DNA and RNA ligand binding ability of TFIIIA. The experimental results also demonstrated that the substitution of zinc fingers 4-7 of TFIIIA with WT1zf1-4 enabled the TFIIIA protein to bind to a specific WT1 RNA ligand (Hamilton et al., 2001). Furthermore, the crystal structures of partial TFIIIA-DNA and TFIIIA-RNA complexes have been solved. The crystallography data indicates that the \( \alpha \) helices within certain zinc fingers make contacts with specific bases in both DNA and
RNA (Nolte et al., 1998; Lu et al., 2003). These findings provided strong evidence that mutations within the α helices would allow for characterization of WT1-ligand interactions. Thus, the previously created Wx+ mutants were employed to investigate the protein interactions of WT1. Each Wx+ mutant had a single α helical region of WT1zf1-4 replaced with the corresponding α helical region from TFIIIAzf4-7. Four mutants were created from WT1 (+KTS) wild type and were represented as Wx+ 1b, 2b, 3b and 4b corresponding to the finger containing the α helical replacement.

To determine the effects made by the α helical swaps, a series of GST pulldown assays were performed. The reactions were carried out using purified His-tagged WT1 wild type and Wx+ mutants and the recombinant GST fusion proteins U2AF65, Ciao 1, par4 and p53. Several independent trials were performed and were analyzed qualitatively by the comparison of relative band intensities between wild type WT1 (+KTS) and the Wx+ mutants. It was expected that a major decrease in band intensity would indicate which zinc finger(s) are critical for WT1 protein-interactions. The results demonstrated that the Wx+ mutants did not cause a significant reduction in the protein binding ability of WT1. A series of pulldown trials varying several buffer components such as salt concentration, detergent concentration, and pH had no effect on the pulldown results.

To confirm the findings were not a result of the WT1 isoform, a similar set of reactions were carried out using WT1 (-KTS). The WT1 (+KTS) isoform contains the KTS insert between zinc fingers 3 and 4 that alters the spacing of the zinc fingers (Haber et al., 1991). This insert disrupts the DNA binding ability of the protein (Haber et al., 1991) and it was considered that the insert may also alter the protein binding ability of WT1. A set of previously made Wx- mutants were employed to determine the effects on
protein binding. Each of the three Wx- mutants had a single \( \alpha \) helical region of WT1zf1-3 replaced by a single \( \alpha \) helical region of TFIIIAzf4-6. The mutants were represented as Wx- 1b, 2b and 3b which corresponded to the zinc finger containing the \( \alpha \) helical replacement. The pulldowns were performed using purified recombinant proteins of WT1 (-KTS) wild type and Wx- mutants and the GST fusion proteins par4, p53 and Ciao 1. The results revealed no significant difference between any Wx- mutant and wild type WT1 (-KTS). From comparisons of WT1 (+KTS) and (-KTS) reactions it was determined that the KTS splice insert was not a contributing factor to the pulldown results.

These unexpected findings gave rise to numerous questions regarding WT1-protein interactions. To develop a clear understanding of why the reactions were not able to duplicated previous findings, the pulldown techniques had to be fully analyzed. The development of the \textit{in vitro} pulldown assay was based on several assumptions. First, the WT1-protein interaction occurs as a direct association of the \( \alpha \) helical regions within the zinc fingers and the protein ligand. Previous literature regarding zinc finger-nucleic acid interactions indicates the \( \alpha \) helices are critical for binding. Therefore, it was assumed that the interaction of WT1 and all its ligands would occur through the \( \alpha \) helices within zinc finger domain. Second, the interaction of WT1 and these protein partners is specific. Although previous studies had identified the protein partners that interact with WT1, possible interactions with other zinc finger proteins were not fully assessed. Third, the WT1-protein interaction involves a single zinc finger of WT1. The association of WT1 and its protein partners may involve multiple zinc fingers as seen with its interactions
with nucleic acids. A drastic decrease in protein binding may not be visualized if compensation in binding is provided by other non-mutated fingers.

4.2 TFIIIAzf4-7

Johnstone and colleagues (1996, 1998) had previously published that the zinc finger protein YY1 does not possess protein binding affinity to Ciao 1 or par4. Despite this finding, other zinc finger proteins had not been assessed for the potential interaction with WT1 protein partners. To determine if the zinc finger donor TFIIIAzf4-7 was able to interact with the WT1 protein partners, several independent GST pulldown assays were performed. From these results it was revealed that indeed an interaction between TFIIIAzf4-7 and Ciao 1 occurs, providing an explanation for the Wx mutants results.

The interaction of the WT1 protein partners with TFIIIAzf4-7 demonstrated the importance of finding a zinc finger donor that did not possess binding ability to these proteins. Several four-zinc finger-containing peptides were screened for their protein interacting abilities. Two peptides of the p43 protein, p43zf1-4 and p43zf6-9, were assayed in pulldown reactions with GST-Ciao 1. Surprisingly, it was found that both peptides displayed an interaction with Ciao 1. Furthermore, interactions were detected with the zinc finger proteins ZAP1, AZF1 and YY1 with Ciao 1. This compilation of results signified that a potential problem was occurring with the pulldown technique. Despite numerous efforts at altering the buffer and reaction conditions no definite conclusions could be made as to why the reactions were unable to duplicate previous findings.
4.3 $^{35}$S-labeled Proteins

The results obtained from the pulldown reactions with the Wx mutants were unanticipated. To determine if the results presented in the literature could be duplicated we attempted a pulldown technique used in previous studies (Johnstone et al., 1996; Johnstone et al., 1998). Three independent trials of pulldown reactions were carried out using in vitro translated $^{35}$S-labeled zinc finger peptides and purified GST fusion proteins.

The pulldown results displayed a significant interaction between WT1 (-KTS) and Ciao 1 and a very negligible interaction between Ciao 1 and YY1, which was consistent with the findings of Johnstone and colleagues (1998). This provided YY1 as a suitable zinc finger donor to continue further analysis into WT1-protein interactions. An interaction was detected between the WD40 protein RACK1 and WT1 (-KTS). This finding was puzzling since Johnstone and colleagues (1998) had previously demonstrated RACK1 did not possess any WT1 binding ability. In addition, the p43zf6-9 peptide displayed a consistent and significant interaction with Ciao 1 in both pulldown techniques.

This discovery that other zinc finger proteins display binding ability to the WT1 protein partners is unexpected but not entirely surprising. Of the previous literature published on WT1-protein interactions, only one group provided a set of experimental negative controls (Johnstone et al., 1996; Johnstone et al., 1998). Considering the functional diversity of zinc finger proteins, the possibility exists that other zinc finger proteins have the ability to bind to the WT1 protein ligands. Although these protein-protein interactions are taking place they are not necessarily the specific, biological
ligands of these zinc finger proteins. In the initial stages of characterizing the DNA binding site of WT1 it was originally discovered that WT1 could bind to the DNA consensus site specific to the zinc finger protein EGR1 (Rauscher et al., 1990). Following extensive studies using mutagenesis and quantitative binding assays it was determined that WT1 does indeed have its own unique DNA binding sequence which it binds with a much higher affinity (Borel et al., 1996; Hamilton et al., 1998). With this in mind, it does seem plausible that the detection of several lower affinity protein-protein interactions may be taking place using this in vitro system. Unfortunately, the pulldown assay cannot be used to calculate differences in binding affinity and binding thermodynamics, thus necessitating the need for quantitative analyses. Further exploration using techniques such as isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR) will allow us to determine the actual affinity of these protein-protein interactions.

4.4 Deletion Mutants and Chimeric Proteins

To characterize which zinc finger(s) are critical in WT1-protein interactions, two deletion mutants and two chimeric proteins were created. The deletion mutants included WT1Δ1, an N-terminal deletion mutant lacking zinc finger 1, and WT1Δ4, a C-terminal deletion mutant lacking zinc finger 4. The deletion mutants were created to provide insight into which zinc finger(s) are important for protein binding and for the creation of additional finger swap mutants. There are several conflicting views in the literature regarding the effects of WT1 deletion mutants on the interactions with RNA and proteins. Caricasole and colleagues (1996) determined that zinc finger 1 is critical for WT1-RNA
binding, while Bardeesy and Pelletier (1998) found the deletion of zinc finger 4 was detrimental to WT1-RNA interactions. Additionally, Davies and colleagues (1998) found that the deletion of WT1 zinc finger 1 produces an increased affinity for U2AF65 in relation to wild type WT1. Unfortunately, the results of this present study were unable to provide any additional information regarding the effects of the deletion mutants on protein binding. The results demonstrated a similar reduction in protein binding to Ciao 1, par4 and U2AF65 for both deletion mutants relative to wild type WT1 (-KTS). The disadvantage to using deletion mutants is that deletion of a full zinc finger may result in the structural instability of the protein. At this time the effects of full zinc finger deletions on the overall folding of the protein are unknown. A more desirable approach involves swapping full zinc fingers from existing, functional zinc finger proteins. This provides the necessary amino acid residues required for proper zinc finger folding and also provides all fingers that may be involved in protein stability.

Using YY1 as a zinc finger donor, two chimeric proteins were created. The W12Y34 chimera contained the first two zinc fingers of WT1 fused to the third and fourth zinc fingers of YY1. The Y12W34 chimera contained the first two zinc fingers of YY1 fused to the third and fourth zinc fingers of WT1. A series of pulldown reactions were performed using Ciao 1, U2AF65 and par4 to determine the relative binding to WT1 (-KTS), YY1, W12Y34 and Y12W34.

A significant decrease in binding to Ciao 1 was revealed for both the W12Y34 and Y12W34 chimeric proteins in relation to wild type WT1 (-KTS). Unfortunately the reduction in protein binding was approximately equal for both chimers and does not provide any conclusive evidence to which zinc finger(s) are more important for protein
interactions. However, this information does suggest that possibly more than one zinc finger is required for WT1-protein interactions. In order to fully investigate this interaction an additional set of mutants including single swap mutants and the replacement of WT1 zinc fingers 2 and 3 would be informative. Very similar results were found for reactions performed with the U2AF65 protein. An equal reduction in U2AF65 protein binding was seen for both chimeric proteins in comparison to WT1 (-KTS) wild type.

The effects of the chimeric proteins were further analyzed in pulldown reactions with the par4 protein. The interactions between both chimers and par4 were reduced in comparison to wild type WT1 (-KTS), although not to the same extent. The interaction between the W12Y34 protein and par4 is significantly less than the interaction between the Y12W34 protein and par4. The replacement of WT1 zinc fingers 3 and 4 caused a drastic decrease in the par4-WT1 interaction. This reduction in binding was not consistent with the results seen for the WT1Δ4 deletion mutant, which caused a minor decrease in binding to par4. Taken together, these results suggest that WT1 zinc finger 3 alone is required for a par4 interaction. Clarification of this finding could be provided by the creation of single zinc finger replacements, in particular the individual substitutions of WT1 zinc fingers 3 and 4. It is interesting to note that the drastic reduction in protein binding caused by W12Y34 is unique to the WT1-par4 interaction. This finding may imply that the interactions between WT1 and different protein ligands involve different zinc fingers. Figure 22 illustrates several possible interactions between WT1 and its protein ligands. A difference in binding stoichiometry for the individual interactions may
Figure 22. Possible interactions between WT1 and its different protein ligands. Differences in binding stoichiometry may result in reduced interactions between the chimeric proteins and some protein partners while abolishing the interaction with other protein partners. The top panel represents two possibilities for protein binding interactions. The bottom panel illustrates the possible effect the chimeric proteins would have on the above binding interactions.
explain the reduced interaction for the chimeric proteins to Ciao 1 and U2AF65 while a loss of binding was found between par4 and the W12Y34 chimeric protein.

The results obtained for two pulldown techniques were inconsistent with each other which raises some interesting points regarding the different techniques. Although both assays were carried out using an *in vitro* approach, the first technique has several additional limiting factors. The initial pulldown assays were performed using highly concentrated, purified proteins which is not consistent with a physiological environment. It is very likely that these conditions may act to overwhelm the reactions or contribute to non-specific interactions between proteins. Furthermore, the proteins were bacterially expressed and purified under denaturing conditions. It is unknown what percentage of the protein has the ability to properly refold and participate in the reactions. It was considered that the reticulocyte lysate system would alleviate some common problems that are found when using *E. coli* expressed recombinant proteins. The *in vitro* transcription and translation of the zinc finger proteins in a eukaryotic cell extract would allow for the proper folding of the proteins, the lysate would contribute a number of other biological proteins to the reaction and the protein of interest would be produced in a lower concentration. The reticulocyte lysate system also contributes endogenous WT1 to the reaction which may act to inhibit some non-specific interactions such as those that may occur with YY1. Although the reticulocyte lysate system provides a more biological setting to study protein-protein interactions, it is still very difficult to conclude how well any *in vitro* system can mimic an *in vivo* environment.

This study did not explore the possible importance of the N-terminal region of WT1 in protein-protein interactions. Previous studies using WT1 C-terminal deletion
mutants identified the zinc finger region as the critical domain in conferring protein binding activity. However, there is some conflicting evidence in the literature that suggests the N-terminus may be involved in WT1-protein interactions (Davies et al., 1998; Richard et al., 2001). It has been proposed that the region encoded by exon 5 is an additional protein-protein interaction site for par4 (Richard et al., 2001). Furthermore, mutagenesis studies have shown that although the zinc finger domain was critically important, the entire WT1 protein was required for a significant interaction with U2AF65 (Davies et al., 1998). In the absence of a WT1 crystal structure, it is unknown if the N-terminus is involved in protein stability, how this region is involved in protein folding or how it affects ligand interactions. Further analysis using full length WT1 may provide some answers regarding the importance of the N-terminal region in these protein-protein interactions.
Chapter 5. Conclusion

WT1 is a key regulatory protein involved in the development of the genitourinary tract. Mutations within WT1 cause multiple syndromes with the most severe effects being Wilms’ tumor, a pediatric kidney cancer. The functional diversity of WT1 can be seen through its interactions with a wide range of ligands, such as DNA, RNA and proteins. The interaction between WT1 and DNA has been well characterized, while the interactions with RNA and proteins still require intensive investigation. A diverse group of WT1 protein partners have been identified but the characterization of the protein-protein interactions has been limited. The use of C-terminal truncation mutants have determined the importance of the zinc finger domain in WT1-protein interactions but the specific zinc finger(s) critical for binding have not been revealed.

The present study concentrated on the interactions of WT1 with the protein ligands par4, p53, Ciao 1 and U2AF65. Using site directed mutagenesis several sets of mutant WT1 proteins were created in an attempt to determine which zinc finger(s) are critical in protein binding. The effects the mutant proteins had on the protein interactions were analyzed qualitatively using GST pulldown assays. The results demonstrate a clear role for WT1zf3, and a possible role for WT1zf4 in the WT1-par4 interaction. It was found that the replacement of WT1 zinc fingers 3 and 4 with those from YY1 caused a distinct reduction in binding to par4. This finding was exclusive for the WT1-par4 interaction and may indicate that different WT1-protein partner interactions rely on different zinc fingers. A decrease in protein binding was also apparent for the
interactions between the WT1:YY1 chimeric proteins and the protein partners Ciao 1 and U2AF65. Continued analysis utilizing a quantitative approach will determine the differences in protein binding affinity caused by the WT1 mutant proteins and thereby provide further characterization of WT1-protein interactions.
Literature Cited


