WDR1 and its isoform alter neurite extension in PC12 cells

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UMI
WDR1 AND ITS ISOFORM ALTER NEURITE EXTENSION
IN PC12 CELLS

by Renée N. Tousignant

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2009

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ABSTRACT

WDR1 is an actin binding protein which promotes actin cytoskeletal rearrangement. Our lab has discovered a truncated isoform of mammalian WDR1, which lacks exons 3-5 (WDRΔ35). The function of both WDR1 and WDRΔ35 has not been characterized. The role of WDR1 and WDRΔ35 in neurite extension was examined using qRT-PCR. The WDR1 RNA levels increased during neurite extension in PC12 cells whereas WDRΔ35 RNA levels decreased. Similarly, the overexpression of WDR1 in PC12 cells increased neurite extension whereas WDRΔ35 overexpression decreased neurite number and length. qRT-PCR was also utilized to examine WDR1 and WDRΔ35 RNA levels during mouse brain development. The WDR1 levels decreased in the CNS tissues in adult mice in comparison to earlier stages. The WDR1 levels were 10-15 fold higher in the various tissues throughout development in comparison to WDRΔ35. These findings implicate an important role for WDR1 and WDRΔ35 in neurite extension processes.
ACKNOWLEDGEMENTS

I would like to thank my Supervisor Dr. Andrew Hubberstey for giving me the opportunity to explore research and for his support and guidance throughout the course of my project. I would like to sincerely thank my committee members, Dr. Lisa Porter and Dr. Siyaram Pandey for their help and advice. I would like to acknowledge the Biology Office Staff and Stockroom staff for their friendliness and eagerness to help. I would also like to thank the faculty of Biological sciences and my fellow graduate students for their support and encouragement. I would specifically like to thank Dorota Lubanska, Mohammad Al-Sorkhy and Alex Ward for their tremendous help and support. I would also like to thank those who worked with me in the Hubberstey lab for keeping things fun and interesting. I would like to give a special thanks to my parents for giving me so many wonderful opportunities. I would like to thank my entire family and my friends for all of their love and support in the last couple of years. I would also like to acknowledge the Department of Biological Sciences at the University of Windsor for giving me such a great academic experience.
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LIST OF ABBREVIATIONS

ABP- actin binding protein
AIP1- actin interacting protein 1
Arp- actin related protein
C- carboxyl
CNS- central nervous system
D- aspartic acid or aspartate
DNA- deoxyribonucleic acid
F-actin- filamentous actin
G- glycine
G-actin- globular or monomeric actin
GAPDH- glyceraldehyde 3-phosphate dehydrogenase
GFP- green fluorescent protein
H- histidine
HA- hemagglutinin
Hek- human embryonic kidney
IF- immunofluorescence
IP- immunoprecipitation
kDa- kilodalton
LIMK- LIM kinase
MCS- multiple cloning site
NGF- nerve growth factor
PAGE- polyacrylamide gel electrophoresis
PBS- phosphate buffered saline
PC12- pheochromocytoma
PCR- polymerase chain reaction
PEI- polyethylenimine
qRT- quantitative real time
RA- retinoic acid
RNA- ribonucleic acid
Ser- serine
SDS- sodium dodecyl sulphate
SH-SY- human neuroblastoma cell line
TBS- tris buffered saline
TTBS- tris buffered saline + tween
Tet- tetracycline
W- tryptophan
WASP- Wiskott-Aldrich syndrome protein
WDR1- tryptophan-aspartate repeat protein 1
CHAPTER I

INTRODUCTION

The actin cytoskeleton

The cytoskeleton is one of the most important cellular components. The cytoskeleton provides structural support for the cell and is required for a multitude of cellular processes. The cytoskeleton is made up of three structures, actin filaments (microfilaments), microtubules and intermediate filaments. My study will be focusing specifically on the actin filaments of the cytoskeleton.

For cytoskeletal rearrangement to occur actin networks must be formed and destroyed upon receiving specific cellular signals. Reorganization of the actin cytoskeleton is required for many fundamental cellular events such as meiosis, mitosis, cell migration, neurite extension and many more.

Actin is a 42kDa protein that exists as F-actin filaments and G-actin monomers. The G-actin monomers reversibly polymerize to form F-actin filaments when the concentration of G-actin monomers is above a critical concentration (Sheterline, 1998). The filaments that are formed have a sense of polarity where the newly polymerized end is ATP bound and is called the barbed or plus end. The ATP bound actin is hydrolyzed shortly after polymerization to form ADP actin after passing through an intermediate phase where ADP is bound to an inorganic phosphate, ADP-P_i (Carlier and Pantaloni, 1988). The ADP bound actin forms the pointed or plus end and is opposite to the barbed end where ATP bound actin is found. The ATP bound actin polymerizes much more rapidly than the ADP bound actin since the critical G-monomer concentration required for
polymerization is lower at the barbed end, 0.1uM, compared to the pointed end, 0.6uM (Rickard and Sheterline, 1986). The constant process of gaining actin monomers at the barbed end while losing actin monomers at the pointed end of the actin filament is called treadmilling (Cleveland, 1982; Neuhaus, 1983). Treadmilling is considered to be the greatest contributor to actin turnover in living cells (Carlier, 2003; Pantaloni, 2001; Ono, 2007). Actin turnover is the process of recycling actin monomers and subunits for the creation of new actin networks. The formation of actin networks is dependent on the cooperation between many actin binding proteins (ABPs).

**Actin binding proteins**

Actin turnover is 100-fold slower *in vitro* compared to *in vivo*, which indicates that the process relies on other cellular factors such as ABPs (Zigmond, 1993). A large number of ABPs exist, which act on the actin cytoskeleton in different ways. Some examples of these actin binding proteins are profilin, Arp 2/3, gelsolin, ADF/cofilin, yeast AIP1, and its mammalian homologue, the tryptophan aspartate repeat protein 1, WDR1, which is the main focus of my study.

*Actin interacting protein 1 /WDR1*

AIP1 is an ABP that has been shown to interact with actin and ADF/cofilin through yeast two hybrid studies (Amberg et al, 1995). AIP1 homologues have been identified in *P. polypephalum* (Matsumoto et al, 1998), *D. discoidium* (Aizawa, 1999; Konzok, 1999), *C. elegans* (Ono, 2001), *Xenopus* (Okada et al, 1997), mouse, chick and human (Avital et al, 1999). Yeast AIP1 is homologous to the tryptophan aspartate repeat family of proteins (WDR) in mammals (Avital et al., 1999) and its mammalian
homologue is called WDR1. For this thesis, the terms AIP1 and WDR1 will be used interchangeably depending on the organism being discussed.

WDR1 is a 67kDa and 601 amino acid ABP (Shin et al., 2004). WD repeats were first identified in G proteins and members of the AIP1 family (Avital et al., 1999). The human WDR1 contains 9 WD repeats and therefore 9 beta propeller blades. The Hubberstey lab has discovered a truncated isoform called WDRA35, which is 42kDa, 534 amino acids and lacks exons 3-5. The removal of exons 3 to 5 removes three WD repeats. Beta-propeller structures are composed of WD repeat segments and have been proposed to mediate protein-protein interactions. Therefore the removal of several WD repeats may affect the function of the truncated isoform. WDR1 also contains 6 kelch-like motifs, which have been shown in previous work to bind actin (Adams et al., 1998; Kim et al., 1999; Soltysik-Espanola et al., 1999). Interestingly, the removal of exons 3-5 in the truncated isoform, WDRA35, does not remove any of the kelch-like motifs. The schematic diagram found in Figure 1.1, shows the WD repeats and the kelch-like motifs found in WDR1 and WDRA35. Currently, very little is known about the function of full length WDR1 and there have been no reports on the isoform, WDRA35. Based on WDR1’s interaction with actin, WDR1 may also play a role in cell migration and other fundamental cellular processes. The known function of WDR1 will be discussed in detail following a short description of known actin binding proteins thought to play roles in related actin turnover activities.
Figure 1.1 A schematic diagram of WDR1 and WDRΔ35. The schematic diagram compares the WD repeats (dashed lines) and the kelch-like motifs (black boxes) in full length WDR1 and the truncated WDRΔ35. WDR1 has 9 WD repeats and 6 kelch-like motifs whereas WDRΔ35 has 6 WD repeats but retains all of its kelch-like motifs. The bracket defines the region of WDR1 that is deleted in WDRΔ35.

Fig. 1.1
Other major actin binding proteins

Actin depolymerizing factor (ADF)/cofilin

ADF/cofilin are 15-21kDa ABPs involved in actin filament severing and depolymerization. Many homologues of ADF/cofilin exist in many different species. Some proteins were originally given specific names when first identified such as depactin in starfish and destrin in mammals until studies revealed these proteins all have homologous sequences and similar activities to ADF/cofilin (Reviewed by Ono, 2007). Since this revelation all ADF/cofilin homologues are referred to as ADF/cofilins (Bamburg, 1999). ADF and cofilin have very similar sequences and the lethality caused by the knockdown of ADF in yeast can be rescued by the overexpression of mammalian cofilin (Iida et al, 1993; Meberg and Bamburg, 2000). Most multicellular organisms contain several ADF/cofilin isoforms. The small differences in sequences and activity between ADF and cofilin allow for the ADF/cofilin isoforms in vertebrates to be classified as either ADF or cofilin (Reviewed by Ono, 2007).

There are two actin-binding sites within the ADF/cofilin molecule, the G/F site and the F site. ADF/cofilin is capable of binding G-actin monomers and F-actin filaments (Reviewed by Ono, 2003; Ono, 2007). The G/F site is responsible for binding to both actin monomers and actin filaments and is the site responsible for depolymerization (Ono, 2003). The F site is required for binding to filamentous actin and in severing the filament (Azaiwa, 2001; Ono, 1998). The F site is created when an inorganic phosphate molecule is released towards the centre of the filament (Blanchoin and Pollard, 1999). This loss creates a conformational change in the filament and creates the binding site for
ADF/cofilin (Blanchoin and Pollard, 1999). ADF/cofilin binds ADP-bound F-actin with a 100-fold higher affinity than ATP-bound actin (Carlier et al, 1997). The ADF/cofilin severing occurs when the molecule binds actin and causes a twist in the filament, destabilizing the filament (McGough et al, 1997). ADF/cofilin binding also enhances monomer loss from the pointed end of the actin filament (Carlier et al, 1997). ADF is more effective at depolymerizing actin filaments than cofilin (Chen et al, 2004). Although both ADF and cofilin have much lower severing capabilities compared to another actin filament severing protein, gelsolin (Ono et al, 2004) they are still responsible for increasing the rate of actin turnover by 25 fold (Carlier et al, 1997).

ADF/cofilin is regulated by LIM kinase (LIMK), TES kinase (TESK) and Slingshot, which are downstream of the Rho family of GTPases. The phosphorylation of the serine-3 on ADF/cofilin by TESK (Toshima et al, 2001) or LIMK (Arber et al, 1998; Agnew et al, 1995; Moriyama et al, 1996) inhibits its actin binding capacities in vivo. ADF/cofilin is dephosphorylated by the phosphatase Slingshot, which restores its actin binding activity (Niwa et al, 2002).

ADF/cofilin plays a significant role in actin turnover by increasing the disassembly of actin filaments. Cofilin severing provides free barbed ends where other cofilin monomers can bind and further depolymerize the filament (Ono, 2003). The new barbed ends produced by cofilin severing can promote polymerization by recruiting polymerizing proteins such as profilin or promote depolymerization by the recruitment of proteins which will cap the barbed ends of the filament and inhibit future polymerization such as AIP1/WDR1. The combination of ADF/cofilin and profilin activity increases actin treadmilling by 125 fold (Dirdry et al, 1998).
The severing and depolymerizing activity of ADF/cofilin is important for rapid actin cytoskeleton rearrangement required in processes such as cell migration, cytokinesis and development. During cell migration the cell will form projections called pseudopods. The distal portion of the extending pseudopod is called the growth cone, which consists of actin based structures called lamellipodia and filopodia. ADF/cofilin is localized in the lamellipodia, in the growth cone of migrating cells (Svitkina and Borisy, 1999). The overexpression of ADF/cofilin has been shown to increase the cell motility of Dictyostellium (Aizawa, 1996). Dictyostellium cells overexpressing ADF/cofilin moved two times faster than the control cells regardless of the substrate. ADF is also necessary for cell motility in Listeria (Aizawa et al., 1996; Meberg and Bamburg, 2000; Carlier et al, 1997). A study on the motility of L. monocytogenes showed that ADF increased the rate of actin-based motility in a concentration dependent manner (Carlier et al., 1997). It was proposed by Carlier et al (1997) that this increase in motility was due to ADF’s ability to increase depolymerization of the pointed ends, which is the limiting step in actin turnover.

ADF/cofilin has also been shown to play a role in Xenopus development. It was shown that inhibition of ADF/cofilin in one of the blastomeres at the two-cell stage blocked cleavage of that blastomere (Abe et al, 1996). It is not surprising that ADF/cofilin could potentially play a role in development since they are present in the cleavage furrow of dividing cells (Abe et al, 1996).

The previous studies proposed the potential importance of the presence of ADF/cofilin in actin cytoskeletal processes but these studies also showed that the regulation of ADF/cofilin is just as critical. Abe et al (1996) showed that injecting non-
phosphorylatable cofilin into *Xenopus* blastomeres caused a complete regression of the cleavage furrow where as injection of phosphorylatable cofilin had no effect on cytokinesis (Meberg and Bamburg, 2000). The study also discovered that 30 minutes after the fertilization of *Xenopus* eggs, 60% of the ADF/cofilin was dephosphorylated (Abe et al, 1996). In addition, cell motility in *Dictyostelium*, showed that cell motility did not increase when a mutant ADF/cofilin containing a Glu-3, which can not be phosphorylated in place of the phosphorylatable Ser-3 was overexpressed (Aizawa, 1996).

*Profilin*

Profilin is a 15kDa ABP, first identified in calf thymus (Carlsson, 1976), which binds ATP bound actin monomers in a 1:1 ratio (Jockusch et al, 2007). Profilin is found in prokaryotes and in both invertebrates (Cooley et al, 1992) and vertebrates (Honore et al, 1993; Witke et al, 1998). There is only a small amount of sequence homology between organisms but all profilins contain highly conserved binding domains and functions (Pollard and Quirk, 1994). All profilins contain a site for actin binding (Schutt et al, 1993), an ARP (actin-related protein) site for proteins such as the ABP Arp2/3 (Machesky et al, 1994), a phosphatidylinositol-4,5-biphosphate (PIP2) site (Lassing and Lindberg, 1985) and a site for proteins containing poly-L-proline stretches (Mahoney et al, 1997), which include many different proteins involved in signal transduction. The most studied function of profilin is sequestering actin monomers and polymerizing actin filaments by adding ATP bound monomers to the barbed end of filaments (Pollard and Borissy, 2003; Birbach, 2008; Jockusch et al, 2007). More recently profilin has been shown to also aid in the depolymerization of actin filaments. The exact role of profilin in the cell has not been fully elucidated despite its discovery 30 years ago (Birbach, 2008).
Studies have shown that the loss of profilin blocks murine and bovine development and that silencing of profilin results in a reduction in focal adhesions, cell migration and filament polymerization (Reviewed by Birbach, 2008; Jockusch et al, 2007; Le Clainche and Carlier, 2008).

**Arp2/3**

Arp2/3 is a class of ABPs known for their actin filament nucleating and crosslinking capabilities. Actin nucleation is the clustering of actin monomers that is required for the initiation of actin filament formation. Nucleation is the rate-limiting step in actin filament polymerization. The Arp2/3 complex will speed the process by gathering and nucleating actin monomers to allow for rapid filament polymerization (Mullin, 2000; Welch, 1999). The nucleating activity of Arp2/3 was first identified in *Listeria* (Dramsi and Cossart, 1998). The complex can also bind the pointed end of one filament and the side of another to crosslink the filaments at a 70° angle (Mullins et al, 1998; Mullins, 2000; Welch, 1999). The crosslinking forms a branch-like structure. This branched structure is found in lamellipodia and pseudopodia suggesting that Arp2/3 plays a role in membrane protrusion (Bailly et al, 1999; Machesky et al, 1997; Weiner et al, 1999).

**Gelsolin**

Gelsolin is one of the largest classes of actin severing proteins (Kwiatowski, 1999). Gelsolin is an 85kDa ABP capable of severing actin filaments. Gelsolin will cap the barbed end of the newly severed filament and in turn promote filament depolymerization (Harms et al, 2004; Kwiatowski, 1999). Gelsolin contains six homologous domains called gelsolin-like (G) domains (Kwiatowski et al, 1986). The G1
domain is the domain responsible for most of gelsolin’s severing activity. The G1
domain also prevents polymerization of actin monomers when bound to actin
(McLaughlin et al, 1993). The G1 domain contains a structure very similar to that of the
actin depolymerizing factor (ADF)/cofilin (Hatanaka et al, 1996). Although the structures
are similar, gelsolin and ADF/cofilin do not share similar sequences (Ono, 2007).
Gelsolin binds preferentially to ADP bound actin (Laham et al, 1993, 1995) and will only
sever ADP actin and not the intermediate ADP-Pi (Allen et al, 1996). This preferential
severing is similar to ADF/cofilin’s severing activity (Ono, 2007). Similar to
ADF/cofilin, gelsolin is also regulated by Rac GTPases. The long helix in the G1 domain
binds to actin near the barbed end of the filament competing for binding with profilin
(Schutt et al, 1993) and ADF/cofilin (Wriggers et al, 1998). Studies have shown that the
loss of gelsolin leads to a decrease in motility as well as slower apoptosis in neutrophils
(Kwiatkowski, 1999).

WD repeat proteins and the beta-propeller structure

The WD-repeat family of proteins was originally thought to be unique to
eukaryotes (Garcia-Higuera et al, 1996; Li and Roberts, 2001; Neer et al, 1994).
However, another seven WD-repeat proteins have been identified in prokaryotes (Li and
Roberts, 2001). WD-repeat proteins contain a conserved core sequence, which consists
of approximately 40 amino acids (Garcia-Higuera et al, 1996; Li and Roberts, 2001). The
core sequence is bracketed by a GH (gly-his) located at the N terminus and a WD (trp-
asp) located at the C terminus of the protein (Garcia-Higuera et al, 1996; Li and Roberts,
2001; Neer et al, 1994). The majority of WD-repeat proteins contain 7 repeating core
sequences, which are separated by a variable region varying in length from 6-94 amino
acids (Garcia-Higuera et al, 1996; Neer et al, 1994). Some WD-repeat proteins have as few as four WD repeats and others have as many as 16 (Li and Roberts, 2001). The WD repeats make up a four-stranded antiparallel beta-sheet (Reviewed by Li and Roberts, 2001). Each propeller blade is made up of three strands from one repeating unit along with one strand from the previous beta-sheet (Garcia-Higuera, 1996; Li and Roberts, 2001). The sharing of strands between blades is thought to stabilize the protein (Li and Roberts, 2001). Each beta-propeller is made up of four to eight blades. The specificity of the different WD-repeat proteins is most likely determined by the variable sequence within each protein (Li and Roberts, 2001).

The WD-repeat structure was first identified in the B-subunit of the GTP-binding protein transducin (Garcia-Higuera et al, 1996; Li and Roberts, 2001; Neer et al, 1994). Due to its first identification the repeat has been called the B-transducin repeat, the GH-WD repeat and the WD-40 repeat (Neer et al, 1994). The function of WD-repeat proteins varies greatly. WD-repeat proteins can be involved in signal transduction, transcription, RNA synthesis and processing, cytoskeletal organization, chromatin assembly, vesicular trafficking, cell cycle control and apoptosis (Garcia-Higuera, 1996; Li and Roberts, 2001). There are also many WD-repeat proteins whose functions are currently unknown, including WDR1 and WDRΔ35.

ADF/cofilin’s interaction with AIP1/WDR1

A yeast two hybrid system was used to identify critical binding sites within ADF/cofilin that mediate the interaction with AIP1 as well as with actin (Avital et al., 1999). Mutations in actin that disrupt the binding of ADF/cofilin inhibit the binding
between ADF/cofilin and AIP1. This suggests that AIP1 may form a trimeric complex with actin and ADF/cofilin (Avital et al, 1999, Tsuji et al, 2009). Work done by Avital et al (1999) in yeast, showed that ADF/cofilin and AIP1 require one another for proper localization. (Avital et al, 1999). It was discovered through studies in yeast that AIP1 mutations were not enough to cause lethality in yeast but AIP1 mutations in conjunction with cofilin mutations produced synthetics lethals (Avital et al., 1999; Ono, 2003). This suggests that either the activity of cofilin and AIP1 can compensate for the loss of the other or that when both AIP1 and ADF/cofilin are lost a shared function vital to cell viability is disrupted.

The depolymerization of F-actin filaments by ADF/cofilin occurs with the cooperation of AIP1 (Avital et al., 1999, Kato et al, 2008). It has been unclear until recently whether AIP1 promotes ADF/cofilin depolymerization by capping the barbed end of cofilin-severed filaments or by severing cofilin bound actin filaments. Recent work has suggested that barbed end capping may play a substantial role in ADF/cofilin depolymerization (Kato, et al, 2008; Ono, 2003; Tsuji et al, 2009). Tsuji et al (2009) showed that AIP1 caps barbed ends and prevents polymerization and reannealing of filaments only in the presence of ADF/cofilin (Tsuji et al, 2009). Interestingly, AIP1 will not prevent the polymerization or reannealing of mechanically severed filaments suggesting that AIP1 contains a mechanism to discriminate between ADF/cofilin severed filaments and filaments severed by other proteins or mechanisms (Tsuji et al, 2009).

Further suggesting the requirement of ADF/cofilin for AIP1 activity, the overexpression of LIMK, an inhibitor of ADF/cofilin activity, decreases the barbed end capping and actin binding of AIP1 (Tsuji et al, 2009). AIP1/WDR1 must also have an additional
mechanism for increasing ADF/cofilin severing and disassembly since other barbed end
capping proteins such as gelsolin and cytochalasin B do not increase ADF/cofilin activity
(Ono et al, 2004). However, the mechanism by which this is accomplished has not been
fully elucidated.

Functions of AIP1/WDR1

Studies are beginning to emerge on the role of AIP1 and WDR1 in critical cellular
processes. So far AIP1 and WDR1 have been implicated in a variety of cytoskeletal
processes in many different organisms including yeast, C. elegans, Dictyostelium,
Xenopus, Arabidopsis, Drosophila and mice. Most of the current work has linked AIP1
and WDR1 to processes such as cytokinesis, migration, development, and endocytosis.

AIP1/WDR1 is expressed in the lamellipodia of Xenopus fibroblast cell growth
cones and has been proposed to be largely involved in actin cytoskeletal changes required
for growth cone formation (Tsuji et al, 2009). The nucleation of filaments in the
lamellipodia is necessary for the outgrowth of growth cones. WDR1 is distributed evenly
throughout the lamellipodia and researchers believe it may signal nucleation of filaments
by Arp2/3 and thus aid in the elongation of growth cones in migrating cells or elongation
of neurites (Tsuji et al, 2009)

It was discovered that a knockdown of WDR1 using siRNA in HeLa cells resulted
in impaired cytokinesis leading to an increase in multinucleated cells (Kato et al, 2008).
The knockdown of WDR1 led to an increase in actin filaments during late telophase of
cytokinesis. There was an increase in thickness of the stress fibres similar to the
phenotype when ADF/cofilin and LIMK are depleted (Kato et al, 2008). This suggests
that the results seen may have been due to a decrease in ADF/cofilin disassembly when WDR1 was lost. In *Dictyostelium*, a similar result was described; AIP1 null cells showed a defect in cytokinesis resulting in multinucleated cells in addition to an increase in the duration of cytokinesis (Konzok et al, 1999). The AIP1 null *Dictyostelium* cells also had severely impaired endocytosis, showed an increase in lamellipodial membrane protrusions and had a slower growth rate compared to control cells (Konzok et al, 1999).

Kato et al (2008) studied the role of WDR1 in cell migration. They found that the knockdown of WDR1 in Jurkat leukaemic T-cells decreased cell migration and chemotaxis compared to control cells. The WDR1 knockdown cells showed an increase in membrane protrusions, similar to what was seen in *Dictyostelium* (Kato et al, 2008; Konzok et al, 1999). The number of additional membrane protrusions increased by 23% in the WDR1 knockdown cells and by 43% in ADF/cofilin knockdown cells, compared to the control cells (Kato et al, 2008). The expression of a constitutively active ADF/cofilin rescued the effects of WDR1 knockdown on migration (Kato et al, 2008), further pointing to the potential requirement for the cooperation between ADF/cofilin and WDR1 or the potential compensation of one protein in the absence of the other.

In Xenopus, XAIP1, has been linked to development as it localizes with XAC (Xenopus ADF/cofilin) at the cleavage furrow of blastomeres (Okada et al, 1999). When blastomeres at the two-cell stage were microinjected with large amounts of XAIP1 development was arrested and the localization of XAIP1 and XAC was diffuse compared to before the injection (Okada et al, 1999). Other studies have shown that mutation of *C. elegans* AIP1, (UNC-78), caused severely disorganized actin filaments in the body wall muscle as well as impaired worm motility (Mohri et al, 2006).
In plants, the overexpression of AIP1 in *Arabidopsis* lead to shorter and wider root hair cells but had no effect on stature or leaf structure (Ketelaar et al, 2007). The effects seen in these studies suggest that AIP1/WDR1 may play an important role in cytoskeletal processes by promoting ADF/cofilin disassembly of actin filaments. The loss of AIP1 may reduce the efficiency of ADF/cofilin disassembly and therefore have a negative effect on processes relying on actin turnover.

In mammals, researchers have discovered a pedigree of mice, called redears, which have mutations affecting development and hematopoiesis (Kile et al, 2007). The redears showed evidence of thrombocytopenia, which is a reduction in blood platelets, and inflammation on the ears and tail (Kile et al, 2007). The mapping of the mutation to chromosome 5 and the sequencing of the coding region revealed that the mutation was found in the gene for WDR1 (Kile et al, 2007). The mutation affects the proper splicing of the transcript and results in the loss of 2 amino acids in the C-terminus of the sixth WD repeat of the Wdr1 protein. The homozygous mice for the redears mutation, (Wdr<sup>rd/rd</sup>), had inflammatory lesions develop on their ears and tails 3-6 weeks after birth (Kile et al, 2007). The severity of the lesions directly correlated with an increase of circulating neutrophils, suggesting that a defect in neutrophil function may be the primary cause of the lesions. It was originally thought that the lesions were a result of an autoimmune disease but later screenings proved this was not the case. The researchers also noted that the circulating neutrophils of the Wdr<sup>rd/rd</sup> showed cytoskeletal defects and a decrease in migration. The cytoskeletal defects were due to a decrease in actin depolymerization and the mislocalization of cofilin. This data further reinforces the previous theory that WDR1 is required for proper cofilin localization. The research also showed that Wdr<sup>rd/rd</sup> mice
showed signs of megakaryocytosis. The megakaryocytes, bone marrow cells responsible for platelet production, had abnormal morphology and maturation. The defect in megakaryocyte maturation results in abortive platelet shedding. These are characteristics of macrothrombocytopenia, a disease, which is also found in humans and results in a decrease in mean platelet volume (MPV) (Kile et al, 2007). The work done by Kile et al, 2007 was a unique study linking the loss of wild type WDR1 to clinical disease.

Another study, which implicated WDR1 in clinical disorders, was published by Le Hellard et al (2007). These researchers linked a locus on human chromosome 4 to bipolar affective disorder and schizophrenia. The study focused on 46 individuals from four families with bipolar disorder and/or schizophrenia. They were able to narrow down the region on chromosome 4 related to bipolar disorder and schizophrenia. The region is a 200kb region, which contains the genes encoding BPAD, SLCA9 and WDR1. Therefore, further research will be done to investigate whether a mutation at this locus is the result of bipolar disorder and schizophrenia and whether a mutation in WDR1 plays a role in these disorders (Le Hellard et al, 2007).

The studies carried out so far have all pointed to the fact that WDR1 may be an important regulator of the actin cytoskeleton required for many cellular functions such as migration, development, and cytokinesis. WDR1’s role in these processes may also link the protein to clinical disorders. Most of the work has pointed to the importance of the interaction between WDR1 and cofilin for the proper execution of these processes.

Signaling to the actin cytoskeleton
The Rho family of GTPases, also called GTP binding proteins, is a known regulator of the actin cytoskeleton. GTPases can be activated by many plasma membrane receptors such as the tyrosine kinase receptors, the G-protein coupled receptors and cytokines receptors (Hall, 1994; Hall and Nobes, 2000). The plasma membrane receptors activate guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP with GTP on the GTPase proteins (Hall and Nobes, 2000). The GTP-bound form is the active form of the proteins, which interact with their targets (Hall and Nobes, 2000).

The Rho family of GTPases contains eight members, which share 50-55% amino acid homology (Mackay et al, 1995, Hall and Nobes, 2000). The family is comprised of Rho, Rac, Cdc42, RhoD, RhoG, TC10, Rnd and TTF (Hall and Nobes, 2000; Mackay et al, 1995). Rho, Rac and Cdc42 have all been shown to have affects on the actin cytoskeleton in Swiss 3T3 cells (Mackay et al, 1995).

Rho’s primary role is in the assembly of integrin complexes as well as being responsible for the assembly of actin stress fibres (Mackay et al, 1995). Research has shown that an overexpression of Rho during neurite extension causes the collapse of filopodium and the retraction of the neurite suggesting that Rho may play a role in neurite retraction in contact with a repellant as opposed to outgrowth (Hall and Nobes, 2000; Mackay et al, 1995). As reviewed by Hall and Nobes (2000), Rho also plays an important role in rat embryo fibroblast (REF) cell migration since migration relies on the formation of stress fibres and focal adhesions.

Rac regulates actin polymerization in lamellipodia and membrane ruffling (Nobes and Hall, 1995; Ridley and Hall, 1992). As reviewed by Hall and Nobes (2000), the
inhibition of Rac during a scratch migration assay in REFs completely prevented cell
migration. Cdc42 is involved in filopodia assembly and the inhibition of Cdc42 decreased
cell migration by approximately 50% in REFs (Hall and Nobes, 2000). Work done on
neuritogenesis showed that the continuous activation of Cdc42 lead to the activation of
Rac, forming lamellipodia around the filopodia of the growth cone (Mackay et al, 1995).

It has now been established that the activation of Rho, Rac and Cdc42 has a direct
role on the activation of certain ABPs. The activation of Rac leads to the activation of
gelsolin, through downstream regulators. Rac as well as Cdc42 are upstream regulators
of the actin nucleating family, Arp2/3 (Mullins, 2000).

The activity of ADF cofilin, as mentioned earlier is regulated by LIMK and
Slingshot. Many ABPs share similar signaling pathways and the regulation of these
pathways allows for the coordination of ABPs in order to achieve the proper actin
arrangement necessary for cellular processes which rely on actin reorganization.

Since AIP1/WDR1 are ABPs, which interact with ADF/cofilin, it would seem
likely that a similar signal transduction pathway would also regulate AIP1/WDR1.
However, the regulation of AIP1/WDR1 has not been elucidated.

The actin cytoskeleton and neurite extension

An important cellular process that requires the reorganization of the actin
cytoskeleton is neurite extension. Proper neurite extension is necessary for proper neural
development as well as during neuronal regeneration. During the process of neurite
extension the cell ceases to proliferate and differentiates to form a neuron (Valtorta and
Figure 1.2 Signaling to the actin cytoskeleton. ABPs are critical regulators of the actin cytoskeleton and are regulated through GTPases. Rac is one of the primary regulators of Arp2/3, Gelsolin and Cofilin. Profilin is regulated by a Rho GEF.
Leoni, 1999). The neuron will then begin to extend neurites, which will eventually form either the axon or the dendrites (Valtorta and Leoni, 1999). The rearrangement of the actin cytoskeleton is critical for the extension of neurites. At the tip of the extending neurite is a bulb-like structure called the growth cone and it is primarily made up of F-actin filaments (Mackay et al, 1995; Valtorta and Leoni, 1999). The F-actin filaments in the growth cone are organized into two structures, the lamellipodia and the filopodia. The lamellipodia are flat web-like structures in the centre of the growth cone and their arrangement relies heavily on profilin (Goldschmidt- Clermont et al, 1991; Pantaloni and Carlier, 1993), ARP2/3 (Welch et al, 1997) and ADF/cofilin (Gungabissoon and Bamburg, 2003; Jockusch et al, 2007). The filopodia are finger-like extensions made up of F-actin filaments and are found around the outer edge of the growth cone. The actin filaments in the growth cone of extending neurites are oriented such that their barbed and rapidly polymerizing ends are facing the leading edge. The addition of actin monomers at the leading edge and the depolymerization of the filaments in the centre of the growth cone is the force, which drives the neurites to extend forward (Forscher and Smith, 1988; Gungabissoon and Bamburg, 2003; Lin and Forscher, 1995; Valtorta and Leoni, 1999).

Microtubules are found in the centre region of the growth cone and F-actin filaments accumulate at the end of microtubule bundles, at the leading edge (Valtora et al., 1999). Both microtubules and actin have been shown to rearrange themselves in response to external cues during neurite extension (Valtora et al., 1999).

Actin turnover is very rapid in the growth cone during neurite extension. The addition of cytochalasin B during neurite extension, a drug that prevents the addition of actin monomers to barbed ends, leads to the disassembly of actin networks within the
growth cone (Maclean-Fletcher 1980, Bonder and Mooseker 1986; Gungabissoon and Bamburg, 2003). Since the rearrangement of the actin cytoskeleton is a pivotal factor in neurite outgrowth it is not surprising that many actin binding proteins have also been shown to play a key role in the process.

The role of actin binding proteins in neurite extension

ABPs are important regulators of actin organization and therefore play a fundamental role in the process of neurite extension. The Arp 2/3 family may be important in the process due to their role in forming filament branches especially in the formation of lamellipodia (Svitkina and Borissy, 1999) and filopodia (Svitkina et al, 2003). Profilin is responsible for the addition of monomers at the leading edge and filopodia outgrowth (Geese et al, 2000; Suetsugu et al, 1998). The potential role of WDR1 in the process has not been elucidated. However, a number of studies have looked at the role of its interacting partner, ADF/cofilin and their upstream regulators LIMK and Slingshot.

ADF/cofilin are abundant in neuronal growth cones and the dephosphorylation of ADF/cofilin by Slingshot, activating ADF/cofilin occurs in response to Nerve Growth Factor (NGF) addition in PC12 cells (Meberg et al, 1998). The overexpression of ADF/cofilin in rat cortical neurons results in an increase in neurite extension whereas its knockdown inhibits the process (Meberg and Bamburg, 2000). Similarly, the overexpression of active ADF/cofilin lead to an increase in the length of extending neurites in rat cortical neurons compared to control cortical neurons and cortical neurons expressing inactive ADF/cofilin (Meberg and Bamburg, 2000).
LIMK and Slingshot are regulators of ADF/cofilin and have been implicated in actin reorganization and polymerization (Birkenfeld et al., 2001). LIMK is expressed in many tissues including those within the nervous system (Heredia et al., 2006). LIMK and Slingshot have been implicated in neuritogenesis (Heredia et al., 2006). A study by Endo et al. (2007) showed that both LIMK and Slingshot are abundant in the growth cone of extending neurites. The overexpression of Slingshot in PC12 cells resulted in an increase in neurite extension whereas the overexpression of LIMK in PC12 cells has been shown to inhibit neurite extension (Birkenfeld et al., 2001; Endo et al., 2007). In the study by Endo et al. (2007) the researchers demonstrated that the knockdown of LIMK also results in a decrease in neurite extension. Since LIMK is responsible for inhibiting ADF/cofilin activity through phosphorylation these results suggest that a proper balance between active and inactive ADF/cofilin is necessary for neurite extension.

Objectives

The role of mammalian WDR1 and WDRA35 has yet to be fully determined. Based on previous research, WDR1 has been shown to cooperate with and potentially be a requirement for ADF/cofilin actin depolymerization. The depolymerization and reorganization of actin filaments is important for processes such as cell migration, cytokinesis and development. Research on these processes has implicated WDR1 in the cytoskeletal rearrangements required. To further elucidate the function of WDR1 and WDRA35, my research will focus on whether rat WDR1 and its isoform, WDRA35 play a role in the cytoskeletal process of neurite extension. Determining the role and cooperation of ABPs in neurite extension can give insight into the process of neuronal development and regeneration as well as lead to the discovery of treatments for neurodegenerative
disorders such as Alzheimer’s and ALS. To determine the role of WDR1 and WDRΔ35 in neurite extension the primary objectives of my study will be to:

1. Determine the endogenous WDR1 and WDRΔ35 RNA levels during neurite extension in PC12 and SH-SY cells using qRT-PCR
2. Determine the effect of WDR1 and WDRΔ35 overexpression on neurite number and length during NGF induced neurite extension in PC12 cells
3. Determine the endogenous WDR1 and WDRΔ35 RNA levels in brain tissues during mouse development using qRT-PCR
CHAPTER II

MATERIALS AND METHODS

Cloning HA WDR1 and HA WDRΔ35 into stable cell line vector-pcDNA4/TO

In order to create tetracycline inducible stable cell lines in PC12 cells, vectors needed to be created which contained the WDR1 or WDRΔ35 gene downstream of the Tet operator (TO). Therefore the pcDNA4/TO vector (Invitrogen) was restriction digested for 2 hours at 37°C with 10 units of the restriction enzyme EcoRV (Promega) in a total volume of 100ul. The EcoRV digested DNA was restriction digested with 10 units of the restriction enzyme NotI (Promega) for 2 hours at 37°C. The EcoRV and NotI DNA was then purified using the Sigma GenElute PCR Clean Up Kit.

The pCI-HA-WDR1 and pCI-HA-WDRΔ35 vectors were cut with 10 units of the restriction enzyme Nhel (Promega) in a reaction volume of 50ul for 2 hours at 37°C. In order to fill in the sticky end to create a blunt end for ligation, 15ul of the digested sample was incubated with 2.5units of Klenow fragment, 1mmol nucleotides and 2.5ul Klenow buffer in a total reaction volume of 25ul for 10min at room temperature. The reaction was stopped by heating the solution for 10 minutes at 75°C. The pCI-HA-WDR1 and pCI-HA-WDRΔ35 DNA was then restriction digested with 10 units of the enzyme NotI (Promega) for 2 hours at 37°C in a total reaction volume of 50ul. The HA-WDR1 and HA-WDRΔ35 bands were then excised and the DNA was purified from the gel using the Sigma GenElute Gel Extraction Kit.

The EcoRV and NotI digested pcDNA4/TO vector was then ligated with the HA-WDR1 and HA-WDRΔ35 inserts using 0.5 units of ligase. The ligation reaction
proceeded at 15°C for 2 hours. The ligation products were then transformed into DH5-alpha *E. coli* cells using 100ul of TCM buffer (50mM CaCl₂, 30mM MgCl₂, 10mM Tris pH 7.5). The transformations (400ul) were streaked out onto LB/ampicillin (50mg/ml) plates. Twelve colonies were selected from each plate and digested with 10 units of EcoRV (Promega) and 10 units of NotI (Promega) for 2 hours at 37°C. One clone for pcDNA-HA-WDR1 and pcDNA-HA-WDRΔ35 was selected and grown up in LB/ampicillin (100ug/mL) and the plasmid was isolated using the Sigma HP Plasmid Maxi Prep Kit. These vectors will be referred to as pcDNA-HA-WDR1 and pcDNA-HA-WDRΔ35.

Expression verification of pcDNA-HA-WDR1 and pcDNA-HA-WDRΔ35 vectors

The successful cloning of the pcDNA-HA-WDR1 and pcDNA-HA-WDRΔ35 vectors was verified by transfecting the vectors into TRex Hek 293 cells (gift from Dr. J. Hudson, University of Windsor). This cell line was selected since it expresses the Tet repressor, which is required to repress the expression of the vectors in the absence of Tet. 8ug of pcDNA-HA-WDR1 and pcDNA-HA-WDRΔ35 was transfected into TRex Hek 293 cells using PEI (2mg/mL in water) as the transfection reagent at a concentration of 10ug/mL media. The media was replaced after 24 hours and 1ug/mL doxycycline (Clontech) was added to one of each of the pcDNA-HA-WDR1 and pcDNA-HA-WDRΔ35 plates. After 24 hours the protein was extracted in 100ul radioimmunoprecipitation, (RIPA) buffer (20mM Tris, 150mM NaCl, 10mM KCl, 1% NP-40).
The protein extracts were incubated with 50ul protein A beads (Sigma) crosslinked to the mouse anti-HA antibody for 2 hours at 4°C (see below). 40ul of 5x sample buffer was added to each extract and the extracts were boiled for 5 minutes. Approximately 20ug of protein was loaded on a 10% SDS-polyacrylamide gel using 1X Tris-glycine running buffer (5X stock- 25mM Tris base, 250mM glycine). The 5X stock was diluted into a 1X solution and 0.1% SDS was added. The protein was then transferred to nitrocellulose membrane (GE Water and Process Technologies) for 1 hour at 100 volts in 1X transfer buffer (14.4g glycine, 3.02 Tris-base and 20% methanol).

The membrane was blocked with 5% skim milk in 1 X TTBS overnight. 1X TTBS is made by diluting a 10X TBS stock (79g Tris, 88g NaCl in 1L H2O pH 7.5) and adding 0.05% Tween (Fischer Scientific). The membrane was then probed with a mouse 1° anti-HA antibody at a concentration of 1:10 000 for 1 hour. The membrane was washed in 1X TTBS 3X for 5 minutes and then probed with a goat anti-mouse 2° antibody conjugated to horseradish peroxidase (Molecular Probes) at a concentration of 1:4000. A total of 10 ml of substrate (Roche Lumi-Light Western Blotting Substrate) was added for 1 minute before imaging. The membrane was imaged using the Alpha Innotech imaging system.

The anti-HA 1° antibody was crosslinked to 1mL protein A beads by first mixing the antibody with the beads for 1hr at room temperature. The beads were then washed 2x in 10 volumes of borate buffer (0.2M Na-borate pH9.0). The beads were then resuspended in 10 volumes of borate buffer and solid dimethylpimelimidate (DMP) was added to a final concentration of 20mM and mixed for 30min at room temperature. The
reaction was stopped by washing the beads 2x in ethanolamine (pH 8.0) at room temperature. The beads were then washed and stored in 1x PBS.

Cell culture

Rat PC12 cells (ATTC) were grown on collagen coated plates (0.1mg/mL rat tail collagen (BD Biosciences, Bedford, MA) in 0.02M acetic acid solution). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma) and 1% penicillin/streptomycin antibiotics (Sigma) at 37°C in 5% CO2. PC12 cells stably expressing the tetracycline repressor (hereafter called PC12-TR) were cultured as described above with the exception of the addition of 2.5ug/mL blasticidin (Invivogen), allowing for stable selection (described below). The PC12-TR stables cell lines expressing the HA or GFP tagged WDR1 and WDRΔ35 were cultured as above and selected for stable integration by the addition of 2.5ug/mL blasticidin (Invivogen) and 250ug/mL zeocin (Invivogen).

SH-SY-5Y cells (ATCC) were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin antibiotics (Sigma) at 37°C in 5% CO2. TRex cells (gift from Dr. J. Hudson) were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma), 1% penicillin/streptomycin antibiotics (Sigma) and 5ug/mL blasticidin (Invivogen) at 37°C in 5% CO2.

Creating PC12 stable cell lines
The tetracycline inducible stable cell line system requires the expression of the Tet repressor in the cells therefore the PC12 cells required the stable integration of the Tet repressor (TR) through the transfection with the pcDNA-TR vector (Invitrogen). The pcDNA-TR vector was transfected into PC12 cells on 10 cm plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The cells were selected for stable integration of the vector using 2.5ug/mL blasticidin (Invivogen). Cells were tested for the stable expression of the tetracycline repressor by transfection with a pcDNA-Lac Z vector (Invitrogen), using PEI as the transfection reagent at a concentration of 10ug/mL. Doxycycline (Clontech) was added at a concentration of 1ug/mL to the plates, 24hr after transfection to induce the expression of the lac Z gene. The cells were fixed after 24hrs with 0.05% glutaraldehyde (Sigma) solution for 5 minutes and stained with 1mg/mL Xgal. A successful PC12 cell line expressing the tetracycline repressor was selected based on the number of blue cells on each plate. This cell line will now be referred to as the PC12-TR.

The pcDNA-HA-WDR1 and pcDNA-HA-WDRA35 vectors mentioned above and pcDNA-GFP, pcDNA-GFP-WDR1 and pcDNA-GFP-WDRA35 vectors (previously constructed in the lab) were transfected into the PC12-TR cells. The transfection was carried out on 10cm plates using Lipofectamine 2000 (Invitrogen) as per the manufacturer’s protocol. In order to select for the stable integration of the vectors, zeocin (Invivogen) was added to the PC12-TR cells at concentrations of 250ug/mL after transfection. Single antibiotic resistant colonies were selected and grown up on individual plates. The cells that continued to be resistant were induced to express HA-WDR1, HA-WDRA35, GFP, GFP-WDR1 or GFP-WDRA35 by the addition of 1ug/mL
doxycycline (Clontech) and the protein was extracted and expression was tested using Western blotting. The PC12 cell lines created in this process will now be referred to as PC12 HA-WDR1, PC12 HA-WDRΔ35, PC12-GFP, PC12-GFP-WDR1 or PC12-GFP-WDRΔ35. Table 2.1 provides a summary of the various vectors, proteins and cell lines utilized in the study.

Expression verification of PC12 stable cell lines

Western blotting was performed on protein samples from the PC12-HA-WDR1, PC12-HA-WDRΔ35, PC12-GFP, PC12-GFP-WDR1 and PC12-GFP-WDRΔ35 cell lines to test for expression. The protein was extracted from cells grown on 60mm plates in 100µl RIPA buffer (20mM Tris 150mM NaCl, 10mM KCl and 1% NP-40). The protein was run on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane (GE Water and Process Technologies). The membrane was blocked overnight in 5% skim milk in TTBS. The blot was then probed with either mouse anti-HA 1° antibody at a concentration of 1:10 000 or rabbit anti-GFP primary antibody (Rockland) at a concentration of 1:5000 in 5% skim milk in 1X TTBS for 1 hour. The membrane was then rinsed in 1X TTBS 3X for 5 minutes. The blots were then probed with either the goat anti-mouse (Invitrogen) or goat anti-rabbit (Invitrogen) 2° antibodies conjugated to horseradish peroxidase (HRP) at a concentration of 1:4000 in 5% skim milk in 1X TTBS for 1 hour. The HRP detection was accomplished as described previously. GAPDH was used as a loading control therefore the membrane was subsequently stripped for 30min using Restore Western Blotting Stripping Buffer (Thermo Scientific). The membrane was then probed with mouse anti-GAPDH 1° antibody (Ambion) at a concentration of 1:5000 in 1X TTBS for 1hour. The membrane was then rinsed with 1X TTBS and
Table 2.1 Summary of the vectors, proteins and cell lines utilized.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Cell Line</th>
<th>Protein</th>
<th>Utilized in Cloning</th>
<th>Utilized in Stable Cell Line Creation</th>
<th>Number of Cell Lines Created</th>
<th>Utilized in Neurite Extension Assays</th>
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<tr>
<td>pCI-HA</td>
<td>-</td>
<td>HA-WDR1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pCI-HA</td>
<td>-</td>
<td>HA-WDRΔ35</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pcDNA-TO</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>PC12</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>5</td>
<td>+ (1)</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>pcDNA-TO</td>
<td>PC12</td>
<td>GFP-WDR1</td>
<td>-</td>
<td>+</td>
<td>5</td>
<td>+ (1)</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
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<td>+ (1)</td>
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</tbody>
</table>
probed with goat anti-mouse 2° antibody conjugated to HRP at a concentration of 1:4000 in 1X TTBS for 1 hour. The membrane was imaged using the Alpha Innotech imaging system.

Measuring endogenous WDR1 and WDRΔ35 RNA levels in PC12 cells during neurite extension

Endogenous WDR1 and WDRΔ35 RNA levels in normal PC12 cells were analyzed after the addition of Nerve Growth Factor (NGF). The PC12 cells were cultured as previously described on 60mm plated until 80% confluent. Differentiation media was added (DMEM + 0.25% Horse Serum) for 24 hours. The media was replaced with fresh differentiation media and 100ng/mL NGF 2.5S (Sigma) was added. RNA was extracted at several time points (0hr, 1hr, 3hr, 6hr, 12hr, 1d, 3d, 5d) after NGF addition using the Sigma Gene Elute Mammalian Total RNA Mini prep Kit. The cDNA was created from 2ug RNA for each sample using ABI High Capacity or the Invitrogen Superscript II Reverse Transcriptase Kit as directed in the manufacturer’s manuals. Quantitative Real Time PCR (qRT-PCR) was performed on each sample in triplicate or quadruplicate. A Master Mix was made up containing the TaqMan Master Mix, Rat WDR1 probe or WDRΔ35 probe along with Rat GAPDH probe as an internal control and the GAPDH forward and reverse primers according to the ABI manual (all qRT-PCR reagents were acquired from Applied Biosystems). The cDNA was added to the 19ul of Master Mix to make up 20ul and the samples were analyzed using an ABI 7300 system. The probe information can be found in Appendix A.
Measuring endogenous WDR1 and WDRA35 RNA levels in SH-SY cells during neurite extension

Endogenous WDR1 and WDRA35 RNA levels in SH-SY cells were measured after the addition of retinoic acid (RA). The SH-SY cells were cultured as previously described on 60mm plated until 40% confluent. RA (10uM) was diluted in 10mM DMSO and then added to each plate at a concentration of 2uM. The RNA was extracted and converted to cDNA as previously described for the PC12 cells. qRT-PCR was then performed on each sample in triplicate. A Master Mix was made up containing the TaqMan Master mix, Human WDR1 probe or Human WDRA35 probe along with Human GAPDH probe as an internal control. (qRT-PCR reagents were purchased from Applied Biosystems). The cDNA was added to the 19ul of Master Mix to make up 20ul and the samples were analyzed using an ABI 7300 system. The probe information can be found in Appendix A.

Neurite extension assay in PC12 cells

The stable PC12-GFP-WDR1, PC12-HA-WDRA35 and a PC12 negative control line (a PC12-TR line that had been transfected with pcDNA-GFP and was zeocin resistant but not expressing GFP) were cultured as previously described on 60mm plates until 80% confluency. The cells were serum starved for 24 hrs then differentiation media (described above) was added to the plates along with 250ug/mL zeocin, 2.5ug/mL blasticidin and 1ug/mL doxycycline (Clontech) was added to induce the expression of GFP-WDR1 and HA-WDRA35. The NGF 2.5S (Sigma) was added 24hrs later at a concentration of 100ng/mL. The cells were imaged 3hrs, 1d, 2d, 4d and 7d after NGF addition using a
Zeiss Aziovert 40 CFL microscope and the Q Capture 2.90.1 software. The number of cells bearing neurites was counted from approximately 7 images of each plate and a percentage was given for the PC12-GFP-WDR1, PC12-HA-WDRA35 and negative control line based on the number of cells bearing neurites compared to the total number of cells. Cells considered to be bearing neurites were those with neurites at least half the length of the cell body. The images were also used to measure the average and median length of the neurites. The neurite lengths were measured using the Q Capture Pro 5.1.1.14 software. The number of neurite bearing cells, the average neurite length and median neurite length for PC12-GFP-WDR1 and PC12-HA-WDRA35 was compared back to the negative control cells. The results achieved were obtained from the data collected from 3 separate experiments.

Microscopy

In order to verify that the PC12-GFP-WDR1 and PC12-HA-WDRA35 cells were overexpressing the WDR1 and WDRA35 constructs fluorescence microscopy and immunofluorescence was utilized, respectively. The PC12-GFP-WDR1 cell line was analyzed under a Zeiss Aziovert 40 CFL fluorescence microscope to verify the induced expression of GFP-WDR1 and the cells were images using the Q Capture 2.90.1 software. The PC12 HA-WDRA35 cells were plated on collagen-coated coverslip. The cells were fixed on the coverslips in 3.7% formaldehyde, permeabilized in Triton-X-100 (Foscher Biotech) and rinsed in 1X PBS. The 1° mouse anti-HA antibody is added to the coverslips at a concentration of 1:100 and incubated at 37°C for 30min. The coverslips were rinsed in PBS + 0.5% Tween 20 (Fischer Biotech) and incubated with the anti-mouse 488nm Alexa 2° antibody at 37°C for 30mins. After the incubation the coverslips
were rinsed in PBS +0.5% Tween 20 (Fischer Biotech) and 1X PBS and placed on slides with a small amount of Prolong Antifade. The slides were analyzed using a Zeiss Azioskop Z mot plus microscope and imaged using the Northern Eclipse software.

**Measuring WDR1 and WDRA35 expression levels in nervous tissue during mouse development**

The TissueScan Mouse Developmental Tissue qPCR Array was employed (Origene). A mastermix was made up with 2X Taqman mastermix and the 20X mouse WDR1 probe (ABI) as per the Origene protocol. 30ul was aliquoted into each well, which already contained dried cDNA from various CNS tissues. The cDNA was allowed to dissolve by placing the plate on ice for 15min. The 96 well plate was then loaded into the ABI 7300 system and the samples were analyzed. The same experiment was run on a separate TissueScan plate using the probe against WDRA35.

The GAPDH levels were also examined to serve as a control. A master mix was made up with 2X Taqman master mix, the forward and reverse GAPDH primers (10X) and the rodent GAPDH probe (20X). The plate was analyzed using the ABI 7300 system. The probe information can be found in Appendix A.

The relative quantifications were determined manually since the control and the target were not analyzed at the same time. The first step was to subtract the GAPDH C_T (threshold cycle) from the WDR1 C_T or WDRA35 C_T for each sample. This results in the Δ C_T value. The Δ C_T value was then subtracted from the Δ C_T value of the sample chosen as the calibrator, which was either the WDR1 13day embryo midbrain sample or the WDRA35 13day embryo midbrain sample. This gives the ΔΔC_T value. The relative
quantification for each sample can then be determined by using the equation; \( RQ = 2^{-\Delta \Delta Ct} \).

The log of the RQ was plotted for each sample. Another graph was plotted comparing the WDRΔ35 \( C_T \) to the WDR1 13day embryo midbrain \( C_T \) value allowing for a better comparison between the WDR1 and WDRΔ35 expression levels.
CHAPTER III
RESULTS

Creating a tetracycline-inducible PC12 stable cell line system expressing WDR1 and WDRA35

To examine the effect of WDR1 and WDRA35 overexpression on the process of neurite extension, an inducible PC12 stable cell line system was produced. This system allowed for the inducible expression of GFP or HA tagged WDR1 and WDRA35 in PC12 cells under the control of the tetracycline operator, only in the presence of tetracycline. The first step in creating this system required the integration of the Tet repressor into the PC12 cells. Once the PC12 cells had been transfected with the Tet repressor and resistant colonies were selected the successful expression of the Tet repressor needed to be verified. This was done by transfecting the pcDNA-LacZ vector into the PC12 cells. The Lac Z gene is under the control of the Tet operator and will be repressed if the Tet repressor is present before the addition of Tet and should then be expressed after the addition of Tet. Fig 3.1 shows images of the PC12 cells after the Lac Z transfection with and without Tet. Figure 3.1 shows an increase in blue colonies due to an increase in Lac Z expression in the PC12 cells after the addition of Tet. The increased number of blue colonies in the PC12 +Tet cells indicates the successful integration of the repressor and the ability of the Tet repressor to inhibit expression in the absence of Tet. Fig. 3.1 also shows the presence of blue colonies in the PC12 - Tet cells suggesting that the system does not tightly regulate protein expression and some leakiness is present. The PC12 cells with the successful integration of the Tet repressor will be referred to as PC12-TR.
Figure 3.1 The tetracycline repressor was successfully integrated into PC12 cells. The PC12 cells transfected with the lac Z gene -Tet had fewer blue colonies compared to the +Tet cells showing repression by the Tet repressor and the successful expression of the Tet repressor in the PC12-TR cells.

PC12 cells (- Tet)

PC12 cells (+ Tet)

Fig. 3.1
The second step in creating the system required the cloning of HA tagged WDR1 and WDRΔ35 into the multiple cloning site of the inducible pcDNA4/TO vector. After successful cloning, the vectors were transfected into T-Rex Hek 293 cells and protein samples were extracted and analyzed using SDS-PAGE analysis. The western blots in Figure 3.2 show the proper expression of HA-WDR1 at 60kDa and HA-WDRΔ35 at 42kDa in the T-Rex cell line upon the addition of tetracycline. The results of the western blots indicate that the induced expression of HA-WDR1 and HA-WDRΔ35 is possible using these vectors and that the cloning was successful. Figure 3.2 also shows the presence of HA-WDR1 and HA-WDRΔ35 bands in the absence of Tet suggesting that there is some leakiness in the system and that small amounts of HA-WDR1 and HA-WDRΔ35 are being expressed in the absence of Tet.

In order to complete the stable cell line system the PC12-TR cells were transfected with the pcDNA-HA-WDR1, pcDNA-HA-WDRΔ35, pcDNA-GFP, pcDNA-GFP-WDR1 and pcDNA-GFP-WDRΔ35 vectors. Protein was extracted from the five different cell lines in the presence and in the absence of Tet. The protein was analyzed using SDS-PAGE analysis to verify the successful integration and inducible expression of GFP-WDR1 and HA-WDRΔ35 in the PC12-TR cells. Figure 3.3A shows the successful integration and expression of GFP-WDR1 into the PC12-TR cells by the presence of a band at 90kDa. Figure 3.3B shows the successful integration and expression of HA-WDRΔ35 based on the presence of a band at 42kDa. The western blots in Fig 3.3A and B also show the increased expression of GFP-WDR1 and HA-WDRΔ35 upon the addition of tetracycline based on the increased intensities of the bands.
Figure 3.2 The HA-WDR1 and HA-WDRA35 constructs were successfully expressed in T-Rex Hek 293 cells. The proper expression of the pcDNA-HA-WDR1 or pcDNA-HA-WDRA35 constructs in T-Rex Hek 293 cells was examined using SDS-PAGE analysis. The arrows indicate the WDR1 (60kDa) and the WDRA35 (42kDa) bands. The intensities of the bands show that both HA-WDR1 and HA-WDRA35 are expressed at a higher level in the presence of Tet compared to in the absence of Tet.
Figure 3.3 *PC12* stable cell lines were created that expressed GFP-WDR1 and HA-WDRΔ35 upon the addition of tetracycline. The proper expression of GFP-WDR1 and HA-WDRΔ35 in the PC12-GFP-WDR1 and PC12-HA-WDRΔ35 stable cell lines was examined using SDS-PAGE analysis. A. The presence of a band at 90kDa shows that GFP-WDR1 is being expressed in the PC12-GFP-WDR1 stable cell line. The band intensities of the blot show the increased expression of GFP-WDR1 in the presence of Tet compared to in the absence of Tet. B. The presence of a band at 42kDa indicates the successful expression of HA-WDRΔ35 in the PC12-HA-WDRΔ35 stable cell line. The band intensities show that there is an increase in HA-WDRΔ35 expression in the presence of Tet.

A.

![GFP-WDR1 Control vs GFP-WDR1 +tet vs GFP-WDR1 -tet](image)

GFP-WDR1 (anti-GFP) →

GFP-WDR1 Control

GFP-WDR1 +tet

GFP-WDR1 -tet

92

B.

![HA-WDRΔ35 +tet vs HA-WDRΔ35 -tet](image)

HA-WDRΔ35 (anti-HA) →

HA-WDRΔ35 +tet

HA-WDRΔ35 -tet

-42

**Fig. 3.3**
These results show that two inducible PC12 stable cell lines were successfully created to express GFP-WDR1, referred to as the PC12-GFP-WDR1 and to express HA-WDRA35, referred to as PC12-HA-WDRA35. Both the cell lines created, show leakiness based on the presence of bands in the – Tet lanes on the western blots shown in Figure 3.3A and B. The PC12-GFP, PC12-GFP-WDRA35 and PC12-HA-WDR1 cell lines were not found to express the appropriate constructs.

Figure 3.4A shows the successful integration and expression of GFP-WDR1 in the PC12-GFP-WDR1 stable cell line using fluorescence microscopy. The presence of green cells indicates that the stable PC12 cell line can be induced by the addition of Tet to express GFP-WDR1. Figure 3.4A shows the successful integration and expression of HA-WDRA35 in the PC12-HA-WDRA35 cells using immunofluorescence. There is an increase in HA-WDRA35 expression when Tet is added based on the increased HA detection by the anti-HA antibody which can be seen in Figure 3.4B.

Endogenous WDR1 and WDRA35 RNA levels during neurite extension in PC12 cells and SH-SY cells

In order to gain a better understanding of whether WDR1 and WDRA35 have roles in neurite extension, the change in endogenous RNA levels was measured using qRT-PCR. Figure 3.5 shows the results of one of three separate experiments looking at the expression of WDR1 and WDRA35 RNA levels in PC12 cells during neurite extension. Figure 3.5 shows an increase in WDR1 RNA levels starting at 3h after NGF addition with the greatest increase being at 6h after NGF addition (Fig 3.5). Although each separate experiment did not yield the exact same change in WDR1 RNA
Figure 3.4 *GFP-WDR1 and HA-WDRΔ35 are being expressed in the PC12 stable cell lines* A. Fluorescence microscopy shows the expression of GFP-WDR1 in the PC12-GFP-WDR1 stable cell line. The phase contrast image shows the total cells in the field without fluorescence. B. Immunofluorescence shows the expression of HA-WDRΔ35 in the PC12-HA-WDRΔ35 stable cell line. There is an increase in HA-WDRΔ35 expression when Tet is added.

A.

![PC12-GFP-WDR1 fluorescence](image1) ![PC12-GFP-WDR1 phase contrast](image2)

B.

![PC12 HA WDRΔ35 +Tet](image3) ![PC12 HA WDRΔ35 -Tet](image4)

Fig. 3.4
Figure 3.5 *WDR1 RNA levels increase during neurite extension in PC12 cells.*

The RNA levels of WDR1 and WDRΔ35 were examined in PC12 cells after NGF induction. A representative experiment shows an increase in WDR1 expression starting at 3h with the greatest peak at 6h. The WDR1 RNA levels begin decreasing after 12h. The WDRΔ35 RNA levels decrease 3h after NGF induction and only begin to increase at the 3d time point.
expression level a general trend was observed in the experiment represented here and the other two experiments, which can be found in Appendix B. All three experiments showed WDR1 RNA levels increasing within the first 12 hours of NGF induction and beginning to decrease 1d after NGF addition. Figure 3.5 shows very little change in WDRΔ35 RNA levels over the entire 5day time course. A similar result was seen in the other two experiments (Appendix B), where there was either no change or a decrease in WDRΔ35 RNA levels upon NGF induction. It is currently unknown whether the changes in WDR1 and WDRΔ35 RNA levels reflect changes in protein levels during neurite extension.

Figure 3.6 shows the results of one of two separate experiments looking at WDR1 and WDRΔ35 RNA levels in SH-SY cells during neurite extension. Interestingly, in SH-SY cells, WDRΔ35 expression levels increase whereas WDR1 RNA levels decrease during neurite extension. Figure 3.6 shows an increase in WDRΔ35 RNA levels 1h after RA induction with the greatest increase occurring at 6h. The WDRΔ35 RNA levels begin to decrease after 1d and continue to decrease up until 5d. Figure 3.6 shows a decrease in WDR1 RNA levels 1h after RA addition. The WDR1 levels remained reduced throughout the entire time course. Although the exact level of change is not identical in the second experiment (Appendix C), a similar trend is observed for both the WDR1 and WDRΔ35 RNA levels. The increase in WDRΔ35 and the decrease in WDR1 in the SH-SY cells are exactly opposite to the trends observed in PC12 cells. As mentioned previously, the WDR1 and WDRΔ35 RNA levels may not reflect a change in protein levels.
Figure 3.6 *WDR Δ35* RNA levels increase and *WDR1* RNA levels decrease during neurite extension in *SH-SY* cells. The RNA levels of WDR1 and WDR Δ35 were examined in SH-SY cells after RA induction. The WDR Δ35 RNA levels increase greatly 6h after RA induction and slowly decrease beginning at the 1d time point. The WDR1 RNA levels decrease 3h after RA induction and do not recover during the 5d analysis.
The effect of WDR1 and WDRA35 overexpression on neurite extension in PC12 cells

In order to determine whether the overexpression of WDR1 and WDRA35 had an effect on neurite extension, the PC12-GFP-WDR1, PC12-HA-WDRA35 and PC12-TR negative control cell lines were plated on 60mm plates and differentiation was induced by the addition of NGF. The study was done in triplicate and Figure 3.7 shows a representative image of each cell line taken 3h, 1d, 2d, 4d and 7d after NGF induction.

Figure 3.7 shows an increase in neurite number and neurite length in the PC12-GFP-WDR1 cell line 3h after NGF induction compared to the other cell lines. The images of PC12-HA-WDRA35 show cells with very few neurites at every time point from 3h to 7d.

In order to quantify the effect of WDR1 and WDRA35 overexpression on neurite extension the percentage of neurite bearing cells was determined by comparing the total number of cells and the cells with neurites using the images represented in Figure 3.7. Figure 3.8 shows the percentage of neurite bearing cells for each cell line after NGF addition. Cells were considered to be bearing neurites if the neurites were at least one half the cell body in length. A minimum of 300 cells was counted for each cell line at each time point using the data from three separate experiments. Figure 3.8 shows that the PC12-GFP-WDR1 cells had a higher percentage of neurite bearing cells than both the PC12-TR negative control cell line and the PC12-HA-WDRA35 cell line 3h and 1d after the addition of NGF. After 3h, the PC12-GFP-WDR1 cells had a 21% increase in neurite bearing cells compared to the PC12-TR negative control cells. At 1d the PC12-GFP-WDR1 cells had a 24% increase in neurite bearing cells compared to the PC12-TR
Figure 3.7 *Images showing that overexpression of WDR1 increases NGF induced neurite extension in PC12 cells.* The differentiation of PC12-GFP-WDR1, PC12-HA-WDRΔ35 and PC12-TR negative control cells was induced by the addition of NGF. Images were taken of the PC12 cells at several time points after the addition of NGF. These images are representative of the images that were used to quantify the number of neurite bearing cells, the average neurite length and the median neurite length of the PC12 stable cell lines. There is an increase in neurite bearing cells in the PC12-GFP-WDR1 cells 1h and 3h after NGF addition.

![Images showing neurite extension in PC12 cells](image_url)
Figure 3.8 The overexpression of WDR1 increases neurite number in PC12 cells. PC12-GFP-WDR1 cells show an increase in neurite bearing cells 3h and 1d after NGF addition (**p<0.0001, *p<0.001: t test). By 2d and 4d the number of neurite bearing cells is similar between the PC12-TR negative control cells and the PC12-GFP-WDR1 cells. At 7d the number of PC12-GFP-WDR1 cells bearing neurites decreases whereas the number increases in the PC12-TR negative control cells (**p<0.0001, *p<0.001: t test). The PC12-HA-WDRA35 cells have few cells with neurites after every time point in comparison to the PC12-TR negative control cells (**p<0.0001, *p<0.001: t test).
negative control cells (Fig 3.8). By 2d the PC12-TR negative control cell lines had reached the same percentage of neurite bearing cells as the WDR1 expressing cells (Fig 3.8). By 7d the PC12-TR negative control cells had 29% more neurite bearing cells than the PC12-GFP-WDR1 cells and the PC12-GFP-WDR1 cells had fewer neurites than they had at 4d (Fig 3.8). Figure 3.8 shows that the PC12-HA-WDRA35 cells had a decreased number of neurite bearing cells at 1d and continued to have fewer cells bearing neurites throughout the length of the time course in comparison to the PC12-TR negative control cells and the PC12-GFP-WDR1 cells (Fig 3.8). The number of neurite bearing cells was decreased by 14% at 1d, 31% at 2d, 27% at 4d and 43% at 7d in the PC12-HA-WDRA35 cells compared to the PC12-TR negative control cells (Fig 3.8).

The length of the neurites was measured for each cell line using the images represented in Figure 3.7 at 1d, 4d and 7d after the addition of NGF. Figure 3.9 shows that the PC12-GFP-WDR1 cells had a higher average neurite length compared to the PC12-TR negative control cells and the PC12-HA-WDRA35 cells 1d and 4d after NGF addition. The average length of the PC12-GFP-WDR1 cells was 0.12 um at 1d and 0.4 um at 4d. This was 0.05um and 0.1um longer than the PC12-TR negative control cells at 1d and 4d, respectively (Fig 3.9A). The PC12-HA-WDRA35 cells had a similar average neurite length compared with the PC12-TR negative control cells at 1d (decrease of 0.01um) and 4d (decrease of 0.04) (Fig 3.9A). Figure 3.9A shows that the average length of neurites decreased for both the PC12-GFP-WDR1 and PC12-HA-WDRA35 cells 7d after NGF induction whereas the PC12-TR negative control cells continued to have an increase in average neurite length. The median length of the neurites was also considered. Figure 3.9B shows the PC12-GFP-WDR1 cells had a median neurite length very similar
Figure 3.9 The overexpression of WDR1 increases neurite length in PC12 cells. A. The average length of neurites was determined by measuring the length of neurites from the images of the cells 1d, 4d and 7d after NGF addition. The PC12-GFP-WDR1 cells had longer neurites than the PC12-TR negative control cells and PC12-HA-WDRΔ35 cells after 1d and 4d (*p<0.05, t test). The PC12-HA-WDRΔ35 had neurite lengths similar to PC12-TR negative control cells after 1d and 4d. B. The median neurite length was calculated for each cell line. The PC12-GFP-WDR1 cells had a median neurite length very similar to that if the PC12-TR negative control cells at 1d and 4d. The PC12-HA-WDRΔ35 cells had a median length similar to the PC12-TR negative control cells at 1d and slightly lower than the PC12-TR negative control cells at 4d.
B.

Fig. 3.9
to the PC12-TR negative control cells at 1d and 4d. The PC12-HA-WDRA35 cells had a similar median neurite length after 1d compared to the PC12-TR negative control cells but had a slightly lower median after 4d (Fig 3.9B). Figure 3.9B shows that the median neurite length is decreased in the PC12-GFP-WDR1 and PC12-HA-WDRA35 cells 7d after NGF addition in comparison to the PC12-TR negative control cells.

The effect of WDR1 and WDRA35 overexpression on PC12 cellular morphology during neurite extension

Images of the PC12-TR negative control, PC12-GFP-WDR1 and PC12-HA-WDRA35 cells were taken after the addition of Tet and then again after the addition of Tet and NGF. After NGF has been added and neurite extension has been induced there is a morphological change seen in the PC12-HA-WDRA35 cells that is not seen in the PC12-TR negative control cells or the PC12-GFP-WDR1 cells. Figure 3.10 shows the images of the PC12-TR negative control, PC12-GFP-WDR1 and PC12-HA-WDRA35 cells after the addition of Tet and after the addition of Tet and NGF. After the addition of Tet the images of the three cell lines show cells that are comparable in shape and size. After the addition of Tet the PC12-TR negative control cells and the PC12-GFP-WDR1 cells had similar morphologies where they became more flattened and began to produce neurites. The PC12-HA-WDRA35 cells were larger in size compared to the control cells and the PC12-HA-WDRA35 cells and had a granulated appearance that is not seen in the PC12-TR negative control and the PC12-GFP-WDR1 cells.
Figure 3.10. The overexpression of WDRΔ35 in PC12 cells causes morphological changes after NGF induction. Images of the PC12-TR negative control, PC12-GFP-WDR1 and PC12-HA-WDRΔ cells were taken after the addition of Tet and again after the addition of Tet and NGF. The three different cell lines have a similar morphology after the addition of Tet. The PC12-TR negative control cells and the PC12-GFP-WDR1 cells begin to flatten and produce neurites after the addition of NGF whereas the PC12-HA-WDRΔ35 cells become larger in size and have a granulated appearance.
The expression levels of WDR1 and WDRA35 in nervous tissues at different stages of mouse development

The TissueScan Mouse Developmental Tissue qPCR Array was obtained from Origene, which contains dried cDNA from various brain tissues at 5 different developmental stages. The WDR1 and WDRA35 RNA levels were measured in these different tissues at the different stages and compared. Figure 3.11 shows the WDR1 RNA levels in various neural derived tissues in comparison to the 13day embryo midbrain RNA levels. There is an increase in WDR1 RNA levels in all of the tissues for the 13day, 15day and 18day embryo as well as in the 7day postnatal mouse. Interestingly, the WDR1 RNA levels decrease in the majority of the brain tissues in the adult mouse as compared to embryonic levels (Fig 3.11). However, the WDR1 levels in the medulla are very high in the adult mouse but very low in the 7day postnatal mouse in comparison to the other tissues.

The same experiment was performed looking at the WDRA35 RNA levels in the developmental tissues. Figure 3.12 shows the RNA levels in comparison to the WDRA35 13day embryo midbrain sample. The WDRA35 RNA levels are elevated in the 13day embryo, 15day embryo, 18day embryo, 7day postnatal mouse and in the adult mouse. In Figure 3.12 there is a large increase in WDRA35 RNA levels in the spinal cord in the 18day embryo. There is also a decrease in WDRA35 RNA levels seen in the thalamus of the adult mouse. In order to compare WDR1 levels to WDRA35 levels in the various tissues during development the WDR1 RNA quantification levels were compared to the same calibrator that was used for the WDRA35 RNA level analysis. Figure 3.13 shows
The WDR1 RNA levels decrease in the adult mouse. The WDR1 RNA levels in various brain tissues at different developmental stages were analyzed using qRT-PCR. The WDR1 RNA levels in the various tissues are elevated in the day13, day15, day18 embryo and the day7 postnatal mouse. The WDR1 RNA levels are decreased in the various adult mouse tissues except in the medulla, where a large increase is evident.
Figure 3.12 *WDRα35 is being expressed during mouse development*. The WDRα35 RNA levels are elevated in various brain tissues throughout the 5 developmental stages in comparison to the 13day embryo midbrain sample. There is a large increase in WDRα35 RNA levels in the spinal cord of the 18day embryo. There is also a decrease in the WDRα35 in the thalamus of the adult mouse.
Figure 3.13 The WDR1 RNA levels are much higher than the WDRΔ35 RNA levels during mouse development. The comparison of the WDR1 RNA levels to the WDRΔ35 13day embryo midbrain sample shows that the WDR1 levels are 15 to 20 fold higher than that of WDRΔ35 during development in all of the CNS tissues.
the WDR1 RNA levels in comparison to the WDRΔ35 13day embryo midbrain value. There was a major increase in WDR1 in comparison to WDRΔ35 expression level in all brain tissues at every developmental stage. There is a 15 to 20 fold increase in WDR1 levels in the majority of the tissues at every developmental stage as compared to WDRΔ35.

The results of our study have shown that WDR1 may have a role in the initiation of neurite extension in PC12 cells whereas its isoform, WDRΔ35, may have an antagonistic effect on the process.
CHAPTER IV

DISCUSSION

The proper reorganization of the actin cytoskeleton is crucial for the formation and extension of neurites. This reorganization is regulated by actin binding proteins. Many ABPs such as profilin (Geese et al, 2000; Suetsugu et al, 1998), Arp2/3 (Svitkina et al, 2003; Svitkina and Borisy, 1999) and ADF/cofilin (Meberg and Bamburg, 2000; Endo et al, 1997) have been implicated in neurite extension. WDR1 has been implicated in cellular processes requiring cytoskeletal rearrangements other than neurite extension such as cytokinesis (Konzok, et al, 1999; Kato et al, 2008), cell migration (Konzok, et al, 1999; Kato et al, 2008) and development (Okada et al, 1999) but there have been no studies examining the direct role of WDR1 in neurite extension. In addition, there have been no reports of WDR1's isoform, WDRΔ35 in the literature.

In order to examine whether WDR1 and WDRΔ35 have a role in neurite extension their expression levels were analyzed during neurite extension in PC12 cells using qRT-PCR. The results of this experiment showed that WDR1 RNA levels began increasing as early as 6hrs after NGF induction and dropped rapidly 12hrs after NGF addition and remained low for the remainder of the time course. (Fig 3.5) This suggests a role for WDR1 in the initiation of neurite extension but not in later stages of the process. During the early initiation of neurite extension the growth cone, which is composed primarily of F-actin filaments, must be formed (MacKay, et al, 1995). The F-actin filaments must be oriented so that their barbed ends are facing the leading edge of the extending growth cone. This rearrangement of the actin cytoskeleton upon the addition of NGF requires the
cooperation of many ABPs. It is possible that WDR1 may play a role in the initial rearrangement of the F-actin filaments and is no longer necessary once the filaments are properly arranged. Another explanation for the reduction in WDR1 expression at later time points is that WDR1 is involved in a signaling pathway required for the initiation of neurite extension. Research has shown that many ABPs are regulated through Rho GTPase pathways (Hall and Nobes, 2000). As an example, it has been established that the proper regulation of ADF/cofilin by LIMK and Slingshot is critical for proper neurite extension to be achieved. The signaling pathway for WDR1 has not been established but it has been proposed that WDR1 may signal filament nucleation by Arp2/3 in extending growth cones (Tsuji et al, 2009). It is therefore possible that WDR1 plays a role in a similar signaling pathway as ADF/cofilin and also regulates the activity of unknown interacting partners during the initiation of neurite extension. A western blot examining changes in WDR1 protein levels during neurite extension would further validate the results of the qRT-PCR since it is unsure whether the changes in RNA levels actually reflect a change in protein levels.

Since there are no reports on WDRA35 or its potential function, it was important to determine whether it had a similar function to WDR1 and more specifically whether it was expressed in a similar manner as WDR1 during neurite extension. The results of the qRT-PCR showed that WDRA35 RNA levels was decreased after NGF induction and remained low throughout the entire 5day time course (Fig 3.5). This is the opposite result to what was seen for WDR1. This suggests that WDR1 and WDRA35 have potential opposing roles in the process of neurite formation in PC12 cells.

The WDR1 RNA levels during neurite extension in PC12 cells suggest that WDR1 may be a vital player in neurite extension. In order to further investigate this, the
effect of WDR1 and WDRΔ35 overexpression on neurite extension was examined using PC12 stable cell lines. Figure 3.8 shows the results of WDR1 overexpression on neurite extension. The overexpression of WDR1 increased neurite bearing cells by 24% 3hr after NGF induction (Fig 3.8). The number of neurite bearing cells continued to be higher for the WDR1 overexpressing cells in comparison to the control cells 1day after NGF induction (Fig 3.8). The negative control cells began showing a similar number of neurite bearing cells 2day after induction (Fig 3.8). These findings support the result from the qRT-PCR where WDR1 expression increased shortly after NGF induction and began decreasing 1day after induction. The results of both experiments support a possible role for WDR1 in the initiation of neurite extension, which is supported by the discovery that ADF/cofilin and ADF/cofilin’s regulators LIMK and Slingshot also have roles in neurite extension (Endo et al, 2007; Meberg and Bamburg, 2000; Birkenfeld et al, 2001). The increase in neurite extension as a result of WDR1 overexpression is similar to results found in an experiment on ADF/cofilin, where its overexpression also increased neurite extension (Meberg and Bamburg, 2000). It is not surprising that WDR1 and ADF/cofilin would have similar effects on neurite extension since they are interacting partners and WDR1 is proposed to be necessary for ADF/cofilin actin depolymerization, a process shown to be vital in neuritogenesis (Avital et al, 1999; Kato et al, 2008).

Interestingly, 4 and 7 days after NGF induction, the overexpression of WDR1 reduced the number of neurite bearing cells compared to the control (Fig 3.8). The overexpression of WDR1 decreased the number of neurite bearing cells by 29% compared to the negative control cells, 7days after NGF induction (Fig 3.8). This result is supported by the decrease in WDR1 expression at later time points seen in the qRT-PCR, during neurite extension in PC12 (Fig 3.5) and further supports the notion of WDR1
being necessary only for the initiation of neurite extension. It also suggests that the
expression of WDR1 in later stages of the process may actually inhibit the formation of
neurites or lead to the death of the overexpressing cells. It should be mentioned that the
numbers of live and dead cells were not counted throughout the time course which may
have an effect on the number of neurite bearing cells at later time points.

The overexpression of WDRΔ35 had the opposite effect of WDR1 where the
overexpression of WDRΔ35 decreased the number of neurite bearing cells significantly in
comparison to the control cells. The number of neurite bearing cells remained low 7 days
after NGF induction (Figure 3.8). This supports the qRT-PCR results where a decrease in
endogenous WDRΔ35 expression was seen during the entire course of neurite extension
(Fig 3.6). These results further imply that WDRΔ35 may not be necessary for the process
of neurite extension in PC12 cells and that the overexpression of WDRΔ35 may actually
inhibit the process altogether.

The effect of WDR1 and WDRΔ35 overexpression on neurite length was also
analyzed. The overexpression of WDR1 increased the average neurite length significantly
4 days after NGF induction, in comparison to the control cells (Fig 3.9). However, the
average neurite length in the WDR1 overexpressing cells decreased from 4 to 7 days
whereas the average neurite length of the negative control cells continued to increase (Fig
3.9). This could be due to the overexpression of WDR1 at later stages causing actin
cytoskeletal changes that are not supportive of continued neurite extension. This change
in actin cytoskeleton may be causing a retraction of the neurites. Previous work in the
Hubberstey lab has shown that WDR1 is localized in the lamellipodia 1 day after NGF
induction and moves to the tips of neurites 3 days after NGF induction (Noone and
Hubberstey, unpublished). Based on the current understanding that WDR1 caps the
barbed end of actin filaments in order to increase ADF/cofilin depolymerization (Tsuji et al, 2009; Kato et al, 2008), it is possible that the overexpression of WDR1 at later stages of neurite extension, where actin reorganization begins to slow, results in the inhibition of polymerization at the leading edge by the overabundance of WDR1 capping activity. This increase in WDR1 capping may actually increase ADF/cofilin depolymerization, which would contribute to the collapse of neurites as well. Although ADF/cofilin overexpression has been shown to increase neurite extension (Meberg and Bamburg, 2000), this was only measured 3 days after NGF induction. It would be interesting to examine whether the overexpression of ADF/cofilin at later time points in neurite extension causes a similar result as WDR1 overexpression and begins inhibiting neurite extension and cause neurites to retract or breakdown.

The results of the study also showed that overexpression of WDRΔ35 did not reduce neurite length in comparison to the control cells (Fig 3.9), suggesting that WDRΔ35 overexpression only inhibits the early formation of neurites and if neurites are able to form they will develop normally. It was however interesting to note the morphological change in PC12 cells associated with WDRΔ35 overexpression. The cells became very large and granulated after WDRΔ35 overexpression. This change in PC12 cellular morphology was only observed after the addition of NGF, suggesting that the process of neuritogenesis must be initiated for the WDRΔ35 overexpression to have an effect on morphology. The overexpression of WDRΔ35 may cause a disorganization of the actin cytoskeleton after NGF stimulation. The disorganization may disrupt the actin cytoskeletal changes or prevent actin turnover, which are required for proper neurite extension. This result is interesting because the overexpression of WDR1’s interacting partner, ADF/cofilin has been shown to cause destabilization of actin filaments and
morphological changes during cell cycle progression, another process, which relies on actin cytoskeletal rearrangement (Lee and Keng, 2005). The staining of actin filaments with phalloidin and the examination of the structures using confocal microscopy after NGF induction in the WDR1 and WDRΔ35 overexpressing cells would allow a comparison of filament organization and give an indication as to the effect of WDRΔ35 on actin filament organization. It is also possible that the overexpression of WDRΔ35 may affect the fate of the PC12 cells. PC12 cells can become glial or neuronal in response to different signals. The PC12 cells overexpressing WDRΔ35 have a glial appearance and it is possible that the overexpression of WDRΔ35 leads to a change in cell fate.

The initiation of neurite extension is the first step in neuronal cell development. Evidence suggests that rapid actin cytoskeletal changes are required in the many steps of neuronal development (Jaworski, 2007; Reviewed by Luo, 2002). Since the results of the previous experiments suggested a role for WDR1 in neurite extension, a mouse brain development qRT-PCR panel was utilized to analyze the possible role of WDR1 and WDRΔ35 in neuronal development. The results showed an increase in WDR1 expression in the brain tissues of the day 15 embryo, day 18 embryo and the 7day postnatal mouse in comparison to the day 13 embryo midbrain tissue (Fig 3.11). There was also a higher level of WDR1 expression in the day 13 embryo telencephalon, rhombencephalon and the spinal cord compared to the midbrain (Fig 3.11). Interestingly, the WDR1 levels dropped in the 5week adult mouse in all of the tissues except for the medulla (Fig 3.11). Since brain tissues such as the cerebellum and thalamus finish developing in the first postnatal week (Goldowitz and Hamre, 1998; Evrard and Ropert, 2009) this reduction in WDR1 expression in the adult mouse brain suggests that WDR1 is no longer required
once the majority of neuronal development has occurred and further supports the notion that WDR1 is required for the initiation of neurite formation. The marked reduction in WDR1 levels in the 7 days postnatal medulla and the large increase in WDR1 expression in the 5 week adult medulla is interesting however, the reason for this increase is unknown. It is possible that the adult medulla tissue sample contained a large population of glial cells, which continue to grow and divide in adulthood and perhaps require the expression of WDR1 at later stages.

The expression levels of WDRA35 also increased during mouse CNS development in comparison to the WDRA35 day 13 embryo midbrain (Fig 3.12). However, the WDRA35 expression levels remained elevated in the 5 week adult mouse except in the thalamus. This is unlike WDR1 expression where expression is turned off once adulthood is reached. This then suggests that WDRA35 may not have a similar function to WDR1 during neuronal development. An important finding supporting this idea was in the comparison between the WDR1 levels and the WDRA35 levels in the different developmental tissues. This comparison showed a 15-20 fold increase in WDR1 expression in all of the tissues at every developmental stage including the adult mouse compared to WDRA35 expression levels (Fig 3.13). WDR1 expression levels are much higher than WDRA35 during mouse CNS development. These results strongly support the hypothesis that WDR1 has a greater role in neurite extension and neuronal development than WDRA35.

In order to further examine the role of WDR1 and WDRA35 in neurite extension their expression levels were examined in a human neuroblastoma cell line, SH-SY cells using qRT-PCR. This experiment yielded very interesting results. WDR1 expression was decreased after RA induction whereas WDRA35 expression was increased (Fig 3.6). This
is the exact opposite as to what was seen in the PC12 cells, which suggests one of two possible explanations. First, since the differentiation of SH-SY cells and PC12 cells occurs along different pathways it is possible that the SH-SY pathway utilizes one isoform and the PC12 pathway utilizes the other. Conversely, it is possible that the utilization of the different isoforms is variable in different species and in different tissues. Further work needs to be carried out to verify these hypotheses.

Some difficulty was encountered in the course of the study, which should be mentioned. The creation of the PC12 stable cell lines proved to be difficult due to the low transfection efficiency of the PC12 cells. In order to improve the chances of creating lines expressing the genes of interest, two different transfections reagents were used; Lipofectamine 2000 and P.E.I. and Lac Z tests were done to ensure the transfections were working properly. A total of 49 individual PC12 stable cell lines were transfected with the various constructs and tested. Only two of these lines were successful in expressing the transfected construct, the PC12-GFP-WDR1 and the PC12-HA-WDRΔ35 cell lines. A viral system such as the lentivirus would potentially have been more efficient at delivering the constructs allowing the easier creation of the other cell lines.

The lack of a PC12-GFP control line presented a problem in the neurite extension assay since the results seen in the PC12-GFP-WDR1 cells may have been a result of the GFP expression itself and not WDR1 overexpression. The experiment should really be repeated using a PC12-GFP control cell line. It would also be beneficial to redo the study using cell lines expressing GFP-WDRΔ35 and HA-WDR1. Since the integration of the pcDNA vectors was random it would also be advisable to use more than one PC12-GFP-WDR1 and PC12-HA-WDRΔ35 line to ensure the results seen were not a product of the integration site.
CHAPTER V

FUTURE DIRECTIONS AND CONCLUSIONS

Future Directions

WDR1 and WDR\Delta35 have been shown in this study to modify the process of neurite extension, either by enhancing PC12 neuritogenesis or by inhibiting neuritogenesis. In order to gain a further understanding of the role of WDR1 and WDR\Delta35 in neurite extension the effect of WDR1 or WDR\Delta35 overexpression on the endogenous expression of neural and glial markers could be examined. This has been attempted using antibodies against nestin, a marker for neural progenitor cells and GAP-43, a marker highly expressed in growth cones of developing neurons. However, the experiment was not successful due to low PC12 protein concentrations. This experiment will need to be revised to obtain positive results in the future.

The qRT-PCR showed an increase in WDR1 expression and a decrease in WDR\Delta35 expression during neurite extension in PC12 cells. It would be favourable to have a western blot showing the endogenous WDR1 and WDR\Delta35 protein levels during neurogenesis in PC12 cells. The results of the western blot could potentially further validate the results seen in the qRT-PCR since it is uncertain whether the small increase in WDR1 RNA levels seen in the qRT-PCR would actually translate into an increase in protein levels. This experiment has been attempted however; results were not achieved due to the limited specificity of the WDR1 antibody and the lack of a WDR\Delta35 antibody. In order for this experiment to be completed an antibody would need to be created against an epitope found only in the WDR\Delta35 isoform. This epitope would need to be located at

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the exon 2-6 junction, in order for the antibody to bind only to WDRΔ35 and not WDR1. It would also be necessary to increase the stringency of the anti-WDR1 antibody in order to reduce the background levels.

The results of this study have given support to the role of WDR1 and WDRΔ35 in the process of neurite extension. Quantitative RT-PCR or microarray analysis could be used to measure the WDR1 and WDRΔ35 expression levels in diseased brain tissues from Parkinson’s and Alzheimer’s patients to provide insight into whether deregulation of WDR1 and WDRΔ35 promote the development of certain CNS diseases. It would also be interesting to look at WDR1 and WDRΔ35 expression levels in brain tissue from Schizophrenic patients since WDR1 is located in a region on chromosome 4 that has been linked to bipolar disorders and Schizophrenia (Le Hellard et al, 2007).

Previous work has shown that GTP binding proteins are major regulators of the actin cytoskeleton and actin binding proteins (Reviewed by Halls and Nobes, 2000). ADF/cofilin is regulated by LIMK (Arber et al, 1998; Agnew et al, 1995; Moriyama et al, 1996) and Slingshot (Niwa et al, 2002), downstream effectors of the GTPases Rho and Rac. In order to get a better understanding of how WDR1 effects changes on the actin cytoskeleton it would be useful to determine how WDR1 and WDRΔ35 are regulated. It would therefore be helpful to begin determining whether WDR1 is involved in a similar signaling pathway as ADF/cofilin and if WDR1 has any other interacting partners. The transfection of constitutively active Rho, Rac and Cdc42 would allow the detection of changes in WDR1 and WDRΔ35 expression levels and changes in WDR1 and WDRΔ35’s ability to bind actin and ADF/cofilin in response to the constitutive expression of these regulators. A yeast-two-hybrid system and immunoprecipitation
experiments would also be useful in determining whether WDR1 and WDRΔ35 have other interacting partners in addition to actin and ADF/cofilin such as Arp2/3, which has been proposed in recent work (Tsuji et al, 2009).

In the SH-SY cells, the results of the qRT-PCR showed that WDR1 levels decreased whereas WDRΔ35 levels increased during neurogenesis. In order to support these results it would be beneficial to examine whether WDR1 and WDRΔ35 overexpression affects neurite extension in SH-SY cells. Since there is an increase in WDRΔ35 expression in SH-SY cells during neurite extension it would be expected that WDRΔ35 overexpression would increase neurite extension in SH-SY cells, similar to WDR1 overexpression increasing neurite extension in PC12 cells. The results of this experiment could potentially support the hypothesis that different cell lines or differentiation pathways favour one WDR1 isoform over the other. In addition to examining the effect of WDR1 and WDRΔ35 overexpression on neurite extension it would also be useful to examine the effect of WDR1 and WDRΔ35 knockdown on neurite extension in both SH-SY and PC12 cells. Past research has shown that the knockdown of LIMK decreases neurite formation (Endo et al, 2007) and the knockdown of WDR1 impairs other cytoskeletal based processes such as motility and cytokinesis (Kato et al, 2008; Konzok et al, 1999) so it can be hypothesized that WDR1 knockdown would have a similar effect on neurogenesis.

Conclusions

The process of neurite extension requires rapid reorganization of the actin cytoskeleton. This reorganization is mediated by several actin binding proteins. Many ABPs have been implicated in the process of neurite extension, a key step in neuronal
development. The results of this study have pointed to a potential role for WDR1 in the initiation of neurite extension in PC12 cells and in mouse brain development. WDRΔ35 has been shown to have an opposite effect by its inhibition of neurite extension in PC12 cells and its reduced expression during mouse brain development. Future studies on WDR1 and WDRΔ35 will further our understanding on the roles of WDR1 and WDRΔ35 in neuritogenesis and additional cytoskeletal processes. The determination of key players in the process of neuronal developmental will provide insight into the causes of brain disorders and knowledge for the development of potential treatments.
REFERENCES


### APPENDIX A: qRT-PCR probe information

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<th>Probe Name</th>
<th>Probe ID #</th>
<th>Exon Boundary</th>
<th>Assay Location</th>
<th>Amplicon Length (bp)</th>
<th>Dye</th>
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</thead>
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<td>-</td>
<td>122</td>
<td>vic</td>
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<tr>
<td>Rodent GAPDH (endogenous control)</td>
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</table>
APPENDIX B: PC12 qRT-PCR results
APPENDIX C: SH-SY qRT-PCR results

The graph shows log relative quantification of WDR1 and WDR1d35 at various time points: 0h, 1h, 3h, 6h, 12h, 1d, 3d, and 5d. The x-axis represents time points, and the y-axis represents log relative quantification.
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