Studies on the Expression and Phosphorylation of the USP4 Deubiquitinating Enzyme

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ABSTRACT

The USP4 is a deubiquitinating enzyme found elevated in certain human lung and adrenal tumours. USP4 has a very close relative, USP15, which has caused great difficulty in studying only one or the other. We have had generated two antibodies specific to USP4 and USP15, and have confirmed that the two do not cross react. Although there have been previous findings of interacting partners, possible substrates and pathways in which it is involved, the biological role of USP4 is mostly unknown. We have used these antibodies to determine that USP4 and USP15 expression differs across tissue and cell types, and that expression changes as the organism ages. We have shown that USP4 plays a role in canonical Wnt signaling, perhaps by stabilizing Beta-catenin, and identified GRK2 as a kinase, phosphorylating USP4. These data have provided enough information to form a hypothesis, implicating USP4 with the destruction complex in the Wnt signaling pathway.

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LIST OF ABBREVIATIONS

ADRBK1: Adrenergic, beta receptor kinase 1
Amp: Ampicillin
APC: Adenomatous polyposis coli
BSA: Bovine serume albumin
CK1γ: Caseine kinase 1-γ
CRL: cullin-RING Ub ligase
CSNK: Casine kinase
Cys: Cystein
DMEM: Dulbecco's modified eagle medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic acid
DTT: Dithiothreitol
DUB: Deubiquitinating enzyme
Dvl: Dishevelled
E1: Ubiquitin activating enzyme
E2: Ubiquitin conjugating enzyme
E3: Ubiquitin ligase
EDTA: Ethyl diaminetetraacetic acid
Faf: Fat facets
Fwd: Forward
Fz: Frizzled
GBM: Glioblastoma multiforme
GFP: Green fluorescing protein
Gly: Glycine
GPCR: G protein-coupled receptor
GRK: G protein-coupled receptor kinase
GSK3β: glycogen synthase kinase 3β
GST: Glutathione S-transferase
HAUSP: Herpes virus-associated Ub-specific protease
HEK: Human embryonic kidney
His: Histidine
IP: Immunoprecipitation
IPTG: Isopropylthio-β-D-galactosidase
JNK: jun-N-terminal kinase
K48: Lysine at position 48
kDa: kiloDalton
KCl: Potassium chloride
LB: Luria Bertani broth
LEF: Lymphoid enhancer factor
LqF: Liquid facets
LRP: Lipoprotein receptor-related protein
Lys: Lysine
$MgCl_2$: Magnesium chloride
MHC: Major histocompatibility complex

NaF: Sodium fluoride
$NaVO_4$: Sodium orthovanadate
NaPPi: Sodium pyrophosphate
NEK: Nima-related kinase
NEM: N-ethylmaleimide
NES: Nuclear export signal
Nlk: Nemo-like kinase
NLS: Nuclear localization signal
NSCLC: Non-small cell lung cancer
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
Pgk: Phosphoglycerate kinase
PI: Propidium iodide
PMSF: phenylmethylsulfonyl fluoride
P-Mut: Phosphorylation mutant
Rev: Reverse
RLU: Relative light unit
RNA: ribonucleic acid
RNAi: RNA interference
SDS: Sodiun dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA: short hairpin RNA
siRNA: small interfering RNA
snRNP: small nuclear ribonucleoprotein
TBST: Tris-buffered saline Tween-20
TCF: T-cell factor
Trunc: Truncated
Ub: Ubiquitin
UBP: Ubiquitin specific protease
Unp: Ubiquitous nuclear protein
UPP: Ubiquitin proteasome pathway
USP: Ubiquitin specific protease
WT: Wildtype

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1. INTRODUCTION

1.1 Ubiquitin Proteasome Pathway

Genes within the cell are constantly being transcribed, and the translation, and post-translational modification of their gene products leads to varying levels of abundance of proteins within the cell. However, expression levels of individual proteins rely not only on their rate of production, but also of degradation. There are two proteolytic systems within the cell. Lysosomal degradation, in which a large vesicle known as the lysosome is formed, taking in extracellular proteins and hormones, as well as cell surface membrane proteins to be degraded by lysosomal proteases. The more complex and tightly regulated process which degrades intracellular proteins is known as the ubiquitin proteasome pathway (UPP) (Reinstein and Ciechanover, 2006). Whereas lysosomal degradation occurs exclusively in the cytoplasm and is generally thought to be less specific – although substrates do require a KFERQ-like sequence in order to be transported into the lysosome – proteolysis via the UPP is highly specific and occurs both in the cytoplasm and the nucleus (Dice and Terlecky, 1990; Dice et al., 1990; Jentsch, 1992).

Protein degradation by the proteasome is an important aspect of cellular health. Key functions of the proteasome are to regulate cell cycle progression via degradation of regulatory protein or rate-limiting enzymes, degrade proteins which have not adopted the proper tertiary structure (which would be damaging to the cell) and to generate small peptides that bind to the major histocompatibility comples (MHC) class I molecules that may generate an immune response (Hochstrasser, 1996; Pahl and Baeuerle, 1996; Rock et al., 1994). Given its importance within the cell, it is not surprising that a dysfunctional UPP may be implicated in the pathogenesis of certain diseases. Disorders related to the aberrant

function of the UPP range from neurodegenerative disease, to cancer, to inflammatory diseases, and autoimmunity (Bedford et al., 2009; Hoeller and Dikic, 2009; Liu et al., 2010; Reinstein and Ciechanover, 2006; Weissman, 2001).

One of the key players in the ubiquitin proteasome pathway is a small, 76-amino acid, highly conserved protein known as ubiquitin (Liu et al., 2010). Ubiquitylation is a post-translational modification best known to tag substrates for degradation by attaching to the substrate and creating a zig-zag chain of ubiquitin molecules, linked by their lysine at position 48 (Lys48, or K48) to ubiquitin's C-terminal end (Passmore and Barford, 2004). There are, however, seven lysine residues found on ubiquitin in total, which may create different linkages, sending the substrate protein to a different fate. Substrates bound to a single ubiquitin may recruit specific ubiquitin binding proteins, which lead to downstream signaling effects such as intracellular transport of proteins through late secretory and endocytic pathways, and transcriptional regulation (Hicke, 2001; Hicke and Dunn, 2003; Passmore and Barford, 2004). Substrates bound to a Lys63-linked polyubiquitin chain may be sent for DNA repair, function in the stress response, signal transduction, or protein translation (Galan and Haguenauer-Tsapis, 1997; Hofmann and Pickart, 1999; Passmore and Barford, 2004).

The K48-linked ubiquitin chain is created by linking the glycine found at position 76 (Gly76) on one ubiquitin molecule's C-terminal end to Lys48 on another ubiquitin molecule by an isopeptide bond. Once four ubiquitin molecules have been added to the chain, a threshold is reached wherein the substrate is sent to the 26S proteasome for degradation (Glickman and Ciechanover, 2002; Thrower et al., 2000).

The process of ubiquitylation begins by the ATP-dependent activation of the ubiquitin molecule by a ubiquitin activating enzyme (E1). Ligation of the ubiquitin to either the substrate or another ubiquitin molecule is carried out by a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3) (Hochstrasser, 1996; Thrower et al., 2000). The first step, ubiquitin activation, occurs by the generation of a high-energy thioester bond between the ubiquitin molecule and E1 enzyme, using ATP. These E1 enzymes are highly conserved in evolution, with the human and yeast proteins being 53% identical; the level of conservation over such an evolutionary distance suggests that this is a highly important process. Following activation, the ubiquitin is transferred to an E2 enzyme, where the E3 enzyme, bound to its substrate, can then transfer the activated ubiquitin to an internal lysine residue on the substrate by an isopeptide bond (Gray et al., 2003; Hershko and Ciechanover, 1992). Whereas the E1 enzyme is highly conserved, there are several E2 enzymes, because multiple chain topologies are made. The cell encodes hundreds of E3 enzymes – many different types of proteins must be recognized in order for this system to be useful and efficient (Hochstrasser, 1996).

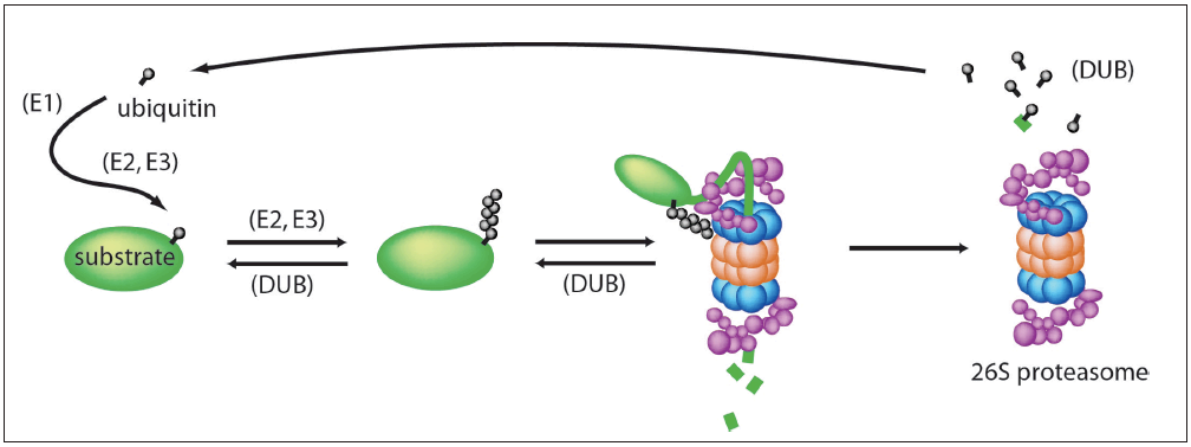
Once the Lys48 ubiquitin chain has been added to its substrate (to its threshold of 4 ubiquitins in the chain), the substrate is targeted to the proteasome for degradation. The 26S proteasome (named so due to its sedimentation coefficient of 26S) is comprised of two smaller complexes: the 20S barrel-shaped proteolytic core, and the 19S regulatory particle. The core particle consists of four stacked seven-membered rings (four rings comprised of seven subunits), of which the subunits are from distinct families: α and β - two β rings sandwiched by the α rings on the outside. The proteolytic active sites are located within the two outside β rings (Groll et al., 1997; Navon and Ciechanover, 2009; Tanaka, 1998). The

19S regulatory particle is composed of at least 19 different subunits, of which 9 form the "lid" and the remaining 10 form the "base" (Adams et al., 1997; Collins and Tansey, 2006; Tanaka, 1998). Collectively, the subunits in the 19S cap function to recognize the ubiquitin chain, unfold it, and guide it down the 20S core, depending on ATP to supply the required energy (Braun et al., 1999). Figure 1 demonstrates schematically the ubiquitin proteasome pathway (Gray et al., 2003).

1.2 Deubiquitinating enzymes

Since the discovery of proteolysis by the proteasome, much progress has been made in characterizing ubiquitin, the enzymes that link ubiquitin to each other and to substrates, and of the proteasome itself. However, our understanding of the enzymes that remove ubiquitin from their substrates and from one another is much less developed. Deubiquitinating enzymes (DUBs), belong to two classes of proteases: cysteine and metallo – although the majority of DUBs are cysteine proteases – which function to hydrolyze the Ub-substrate isopeptide bond (Hussain et al., 2009; Nijman et al., 2005). One such instance is the processing of the ubiquitin gene products; ubiquitin is coded within the genome as fusions. There are four human ubiquitin gene products, UbA52 (ubiquitin fused to a 60S ribosomal subunit), UbA80 (ubiquitin fused to a 40S ribosomal subunit), UbB (a linear fusion of three ubiquitin monomers) and UbC (a linear fusion of nine ubiquitin monomers), all of which must be processed by deubiquitinating enzymes to get the free ubiquitin monomers (Baker and Board, 1991; Finley et al., 1989; Wing, 2003). There are two families of DUBs encoded by all eukaryotic cells: the ubiquitin carboxy-terminal hydrolases (UCHs) and the ubiquitin specific proteases (UBPs, or USPs in mammals) (Wilkinson, 1997). There have been 16

Figure 1: Schematic representation of the ubiquitin proteasome pathway. The E1 ubiquitin activating enzyme, using ATP, generates a high-energy thioester bond between ubiquitin and itself. Following activation, ubiquitin is transferred to an E2 ubiquitin conjugating enzyme, and the E3 ubiquitin ligase, bound to its substrate, is then able to transfer the activated ubiquitin to an internal lysine residue on the substrate by an isopeptide bond. Substrates with the K48 linked ubiquitin chain are sent to the 26S proteasome for degradation. The 26S proteasome unfolds the protein (green line) and sends it through the proteolytic core to be degraded into short peptides (green bars). Deubiquitinating enzymes disassemble the ubiquitin chain into ubiquitin monomers, recycling the small proteins for future use. (Gray et al., 2003)


(E1)
ubiquitin
(E2, E3)
substrate
(E2, E3)
(DUB)
(DUB)
(DUB)
26S proteasome

UBPs and 1 UCH discovered in the yeast *Saccharomyces cerevisiae*, and 50-60 UBPs and 4 (in human) or 5 (in mouse) UCHs identified within the mammalian genome (Soboleva and Baker, 2004).

Ubiquitin carboxy-terminal hydrolases are named so because they process their substrate at the C-terminus of ubiquitin (Chung and Baek, 1999; Pickart and Rose, 1985; Wilkinson, 1997). The UCHs are small enzymes (20-30kDa) with four highly conserved domains spanning approximately 200 amino acids (Chung and Baek, 1999; Wilkinson, 1997). It is thought that their primary role in the cell is to recycle ubiquitin molecules, and to process newly synthesized ubiquitin (Nijman et al., 2005; Pickart and Rose, 1985).

Ubiquitin specific proteases are larger than the UCHs, ranging from 50-300kDa, and although they have little amino acid sequence similarity, all contain the "catalytic triad" of amino acids – a cysteine, histidine and aspartic acid residue required for catalysis, and have similar folding patterns in the USP domain (D'Andrea and Pellman, 1998; Reyes-Turcu et al., 2009). In addition to the catalytic core (comprised of about 450 amino acids), many USPs have N-terminal extensions, and some have C-terminal extensions, which presumably offer substrate specificity or the localization of the enzyme within the cell (D'Andrea and Pellman, 1998; Wilkinson, 1997).

Deubiquitinating enzymes have many functions and play important roles within the cell. Depending on the level of the enzyme within the cell, the DUB may stimulate the degradation or stabilize its protein substrate (D'Andrea and Pellman, 1998). Isopeptidase T (or Ubp14 in yeast) is a very well studied DUB, which promotes degradation of its substrate protein by the proteasome (Amerik and Hochstrasser, 2004; Hadari et al., 1992). In yeast, Ubp14 acts on unanchored ubiquitin chains, releasing free ubiquitin from the unattached

Gly76 residue. Ubp14 (or Isopeptidase T), however, cannot free the ubiquitin chain from a protein substrate; the chain must first be released by Ubp4 (Doa4) (Amerik and Hochstrasser, 2004; Hadari et al., 1992; Soboleva and Baker, 2004). It has been shown that Ubp14 null yeast cells accumulate unanchored polyubiquitin chains and have inhibition of proteolysis. Interestingly, it has also been found that Ubp14 was not essential for growth of the cell (D'Andrea and Pellman, 1998; Wing, 2003). Yeast cells null for *doa4* show an accumulation of ubiquitinated proteins and a reduction in free ubiquitin pools, leading to impaired proteolysis by the proteasome. It has recently been shown that inactivation of genes required for vacuolar proteolysis and endocytosis decreased ubiquitin depletion in *doa4* null yeast cells. It has since been suggested that Doa4 plays a role in the late endosome/prevacuolar compartment, to recycle ubiquitin molecules attached to membrane proteins being trafficked to the vacuole for degradation (Amerik et al., 2000; Soboleva and Baker, 2004; Swaminathan et al., 1999).

It is quite logical that any process in which ubiquitination is involved, one would find deubiquitination close by as well. Thus along with ubiquitin, deubiquitinating enzymes are also important in cell cycle control. One such enzyme is UBPY (USP8). It has been found that UBPY is phosphorylated at Ser^{680}, rendering it catalytically inactive, and bound to 14-3-3 (a regulator protein) during interphase of the cell cycle. During mitosis, UBPY is dephosphorylated, and catalytically active (Mizuno et al., 2007). By increasing or decreasing expression of UBPY, changes in total protein ubiquitination are observed, correlated with cell proliferation; it's accumulation prevents the cell from entering S phase, and its inhibition leads to the accumulation of ubiquitinated proteins on the early endosome,

suggesting that its activity is predominantly located at the endosome (Mizuno et al., 2007; Mizuno et al., 2006; Naviglio et al., 1998; Row et al., 2006).

Deubiquitinating enzymes are also important for development. An example of this is, the product of the fat facets (Faf) gene in *Drosophila*, a housekeeping gene. Faf plays an important role in early eye development (Fischer-Vize et al., 1992). It is thought that Faf's role in eye development is to protect its substrate proteins from degradation by the proteasome. Its substrate has been found to be Liquid facts (Lqf), an endocytic protein. Mutations in Faf result in abnormal development of photoreceptors in the eye, which can be rescued by mutations in the proteasome, thus inhibiting degradation of LqF (Chen et al., 2002; Huang et al., 1995).

Another enzyme responsible for the regulation of expression levels of another protein is HAUSP (herpes virus-associated Ub-specific protease – USP7). HAUSP interacts with the tumor suppressor p53. Low p53 levels are maintained within the cell by Mdm2 – a ubiquitin E3 ligase. HAUSP is able to deubiquitinate p53, protecting it from its degradation (Li et al., 2002). Loss of p53 activity within the cell by mutation is found in approximately half of all cancers, and its apoptotic pathways are impaired in the remaining half (Joerger and Fersht, 2010). This exemplifies that impaired function of deubiquitinating enzymes may play an important (and devastating) role in disease.

There are several other deubiquitinating enzymes that have been found to be associated with human disease. The ubiquitin carboxy-terminal hydrolase, UCHL1 is overexpressed in some tumours, namely lung cancer (Hibi et al., 1998; Hibi et al., 1999). Interestingly, UCHL1 has also been associated with neurodegenerative disorders; Parkinson's disease and Alzheimer's disease. However in these instances UCHL1 is

downregulated and has oxidative modifications (Butterfield et al., 2006; Castegna et al., 2002; Choi et al., 2004). Another DUB with involvement in cancer pathogenesis is the mammalian proto-oncogene, Tre-2. A mutated form of the gene, lacking the His domain, has found to be transforming (Nakamura et al., 1992; Papa and Hochstrasser, 1993).

1.3 Ubiquitin specific protease 4, USP4

USP4, found within the same gene family as *tre-2* (*tre-17* in humans), is a gene discovered in the early 1990's during a survey of genes near the Mpv 20 retroviral insertion site (Gupta et al., 1993). Originally named ubiquitous nuclear protein (Unp), it underwent a name change in 1999 to conform to the new systematic nomenclature for human ubiquitin specific proteases (Baker et al., 1999). The *Unp* gene was first determined to be located on mouse chromosome 9, homologous to the human chromosome 3p21, which has been found to be implicated in many human tumors (Gupta et al., 1993). Deletions or rearrangements at chromosome 3p21 have been shown to occur in tumors of the breast, kidney, colon and lung (Balsara and Testa, 2002; Chen et al., 1994; Hesson et al., 2007; Leris et al., 2005; Senchenko et al., 2004; Tannergard et al., 1994). Shortly after its discovery, localization of USP4 on the human chromosome was further mapped to the 3p21.3 region (Gray et al., 1995). Other genes in this region found to be involved in cancer are RASSF1, a tumour suppressor, which is found to have allelic loss in large cell neuroendocrine tumours of the lung (Pelosi et al., 2010), and RBM5/H37, which is also a tumour suppressor protein found to have decreased expression in lung tumours, and plays a role in altered gene expression in metastases (Oh et al., 2010). USP15, located on human chromosome 12q14 is a very close relative to USP4. USP4 and USP15 share 60.5% sequence identity and 76% sequence

similarity (Angelats et al., 2003; Baker et al., 1999). This high similarity has often made it difficult to study one gene and not the other with high certainty (an issue of relevance to the work that will be described in subsequent chapters).

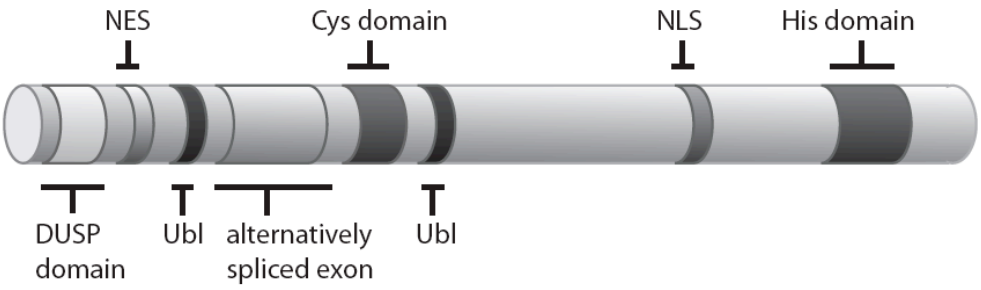
USP4 has been found to be capable of transforming mouse NIH3T3 cells when expressed from a highly active promoter, phosphoglycerate kinase (Pgk) and injected into athymic CD1 mice (Gupta et al., 1994). Since this initial discovery, USP4 expression has been found to be elevated in small cell lung tumors and adenocarcinomas of the lung, and adrenocortical carcinomas (Gray et al., 1995; Laurell et al., 2009; Velazquez-Fernandez et al., 2005).

When first discovered, USP4 was determined to be a nuclear protein (thus why it was originally named Unp), based on its predicted amino acid sequence containing a canonical nuclear localization signal such as that found in p53 (Gupta et al., 1993; Shaulsky et al., 1990). Further experiments showed that upon subcellular fractionation, USP4 indeed fractionates with the nucleus, however another group found that it was localized within the cytoplasm (Frederick et al., 1998; Gupta et al., 1994). In fact, it was more recently found that USP4 not only contains the nuclear localization signal ($_{766}$QPQKKKK$_{772}$) but also a nuclear export signal ($_{133}$VEVYLLELKL$_{142}$), and that its localization within the cell is dependent upon cell type (Soboleva et al., 2005). USP4 is comprised of 22 exons spanning 47.4kb (Di Fruscio et al., 1998). Like other ubiquitin specific proteases, USP4 contains the required conserved amino acids for catalytic activity. The necessary cysteine residue (and therefore termed the 'cys domain') is encoded by exon 8, and the two required histidine residues (the 'his box') is split between exons 20 and 21; both histidines are encoded within exon 20, however exon 21 contains other conserved residues (Di Fruscio et al., 1998;

Hochstrasser, 1996). The USP4 sequence also contains the LxCxE motif which has shown to be a binding site for the tumor suppressor protein, pRb (Taya, 1997). This binding has been experimentally confirmed, and has also shown that USP4 interacts not only with pRb, but pocket proteins p107 and p130, however it is thought that these proteins are not substrates of USP4, but serve more to bring USP4 in contact with other proteins (Blanchette et al., 2001; DeSalle et al., 2001). USP4 has also been found to be phosphorylated at Ser675 and Ser680, and ubiquitinated at Lys171 (unpublished data). The human and mouse USP4 sequences are shown in figure 2, highlighting important regions of the protein.

It has been previously shown that USP4 is able to remove the ubiquitin protein from synthesized human Uba52 as well as natural and engineered ubiquitin-protein fusions and ubiquitin precursors (Gilchrist and Baker, 2000; Layfield et al., 1999). Mutation of the conserved cysteine residue (Cys311) or one of the conserved histidine residues (His880) to alanine results in complete abolishment of the deubiquitinating activity of USP4. Mutation of the second conserved histidine residue (His872) results in a significant reduction in enzymatic activity, albeit not complete abolition, indicating that His880 is most likely the histidine residue found within the catalytic triad (Gilchrist and Baker, 2000). Setting it apart from other deubiquitinating enzymes, USP4 has been found to efficiently cleave the ubiquitin-proline bond, a feat that is either very inefficient or impossible by other DUBs (Gilchrist et al., 1997) with the exception of the closely related USP15. The biological significance of this is unknown (there are no known ubiquitin-proline fusions that occur naturally) but may reflect a large or flexible catalytic cleft that can accommodate the distortion imparted by the proline residue.

Figure 2: Linear depiction of USP4, highlighting important regions of the protein. The nuclear export signal (NES) and nuclear localization signal (NLS) are shown. Exon 7 is alternatively spliced. USP4 contains the conserved USP domain (DUSP) and has two ubiquitin like domains (Ubl). The Cys and His domains form the catalytic active site. Figure designed by Doug Gray.


NES
Cys domain
NLS
His domain
DUSP domain
Ubl
alternatively spliced exon
Ubl

Although the substrates of USP4 remain to be fully elucidated USP4 has been implicated in a number of pathways within the cell. Most recently USP4 was found within the spliceosome wherein it deubiquitinates Prp3, a component of the U4 snRNP (Nottrott et al., 2002). Deletion of USP4 interferes with splicing, cell division, and the cell's proper response to taxol, a chemotherapeutic drug (Song et al., 2010). The authors found that the $Usp4^{Sart3}$ complex regulates the spliceosome by controlling the U4/U6.U5 snRNP (Song et al., 2010). A yeast two-hybrid screen using Ro52 as bait has identified USP4 as a Ro52 interacting protein (Di Donato et al., 2001). It had previously been shown that Ro52, a RING-finger protein belonging to a RBCC (RING-finger/B-box/coiled-coil) family (Torok and Etkin, 2001), is able to ubiquitinate itself and USP4, which is then able to self-deubiquitinate (Wada and Kamitani, 2006). The same group showed that in HEK293T cells, USP4 is able to cleave ubiquitin from Ro52 using its isopeptidase activity, and that it colocalizes with Ro52 to cytoplasmic rod-like structures. It is postulated that the continuous deubiquitination of Ro52 by USP4 may play a role in tumorigenesis (Wada et al., 2006). USP4 has also been found to bind with the A_{2A} adenosine receptor ($A_{2A}R$) protein. USP4 helps $A_{2A}R$ to translocate from the endoplasmic reticulum (ER) to the plasma membrane by binding to the carboxy terminus of the receptor, rescuing it from degradation by the proteasome while it is processed through the golgi apparatus and translocates to the plasma membrane. This increases the amounts of functional receptor to bind ligand and exert a biological response (Milojevic et al., 2006; Toews, 2006). Recently, USP4 has also been implicated in the canonical portion of the Wnt signaling pathway. Using RNAi against various human DUBs, degradation of USP4 was shown to activate B-catenin dependent transcription and that USP4 interacts with two known

Wnt signaling molecules, Nemo-like kinase (Nlk) and TCF4, a transcription factor (Zhao et al., 2009).

1.4 Canonical Wnt Signaling

There are seven members in the G protein coupled-receptor kinase family, which can be divided into three subgroups: the visual GRKs (GRK1 and GRK7), the β-adrenergic receptor kinases (GRK2 and 3) and the GRK4 family (GRK4, GRK5 and GRK6) (Ribas et al., 2007). GRKs are able to recognize, and are allosterically regulated by, activated G protein coupled-receptors (GPCRs), resulting in the recruitment of arrestins, uncoupling of GPCRs from heterotrimeric G proteins, receptor internalization, and the activation of G protein-independent signaling pathways (Boguth et al., 2010; Pitcher et al., 1998; Premont and Gainetdinov, 2007). In addition to the phosphorylation of GPCRs, GRKs have been found to have interactions with non-receptor proteins, demonstrating that GRKs may also have other roles within the cell (Ribas et al., 2007). Interestingly, one of the newly discovered roles of GRK2 is the inhibition of canonical Wnt signaling, a process which USP4 has also recently been connected to (Wang et al., 2009; Zhao et al., 2009).

Wnt signaling is an important cellular process that mediates cell-to-cell communication during both development and adult homeostasis (Clevers, 2006; MacDonald et al., 2009). Wnt signaling can be broken down into two pathways: canonical and non-canonical Wnt signaling. The canonical Wnt signaling pathway involves the multi-functional β-catenin protein, and the non-canonical Wnt signaling pathways are independent of β-catenin, which can be further broken down into the Wnt/jun-N-terminal kinase (JNK) or the Wnt/calcium pathways (Rao and Kuhl, 2010).

Wnt proteins acquired their name by fusing the name of two genes: *Wingless*, a gene discovered approximately 30 years ago in *Drosophila melanogaster*, and the murine gene, *Int-1*, a gene discovered several years later when the mouse mammary tumour virus (MMTV) was discovered to integrate into a common site of the genome, the promoter region of *Int-1* (Giunta, 2009; Nusse et al., 1984; Nusse and Varmus, 1982; Sharma and Chopra, 1976). *Int-1* was determined to be the murine orthologue of the *Drosophila* gene, *Wingless*, and thus the term 'Wnt' was founded (Rijsewijk et al., 1987).

In the presence of these lipid-modified, cysteine-rich glycoproteins, known as Wnts, the canonical Wnt signaling pathway is activated (Giunta, 2009). The Wnt ligands bind to the transmembrane receptor Frizzled (Fz) and its co-receptor LRP6 (or LRP5, a close relative), forming a complex that recruits Dishevelled (Dvl), resulting in the phosphorylation of LRP6 by glycogen synthase kinase 3β (GSK3β) and caseine kinase 1-γ (CK1γ). This phosphorylation of LRP6 recruits Axin, inhibiting Axin-mediated β-catenin phosphorylation, which stabilizes β-catenin, leading to its accumulation and localization towards the nucleus to form complexes with transcription factors T cell factor/lymphoid enhander factor (TCF/LEF) and promote Wnt target gene expression (Clevers, 2006; MacDonald et al., 2009). In the absence of Wnt, cytoplasmic β-catenin is constantly being degraded, preventing it from translocating into the nucleus and activating transcription of the Wnt target genes (MacDonald et al., 2009). Without translocation of β-catenin in to the nucleus, TCF complexes with Groucho/Grg/TLE proteins, inhibiting transcription (Cavallo et al., 1998; Roose et al., 1998). Cytoplasmic β-catenin is degraded by the destruction complex, consisting of the tumour suppressor proteins Axin, and Adenomatous polyposis coli (APC) and the two kinases, CK1 and GSK3α/β (Clevers, 2006). CK1 phosphorylates β-catenin at Ser45, which

is then followed by phosphorylation of Ser33, Ser37 and Thr41 by GSK3β. Phosphorylation of β-catenin at these positions targets the protein to the proteasome for degradation (Rao and Kuhl, 2010). Figure 3 shows a schematic of the canonical Wnt signaling pathway (MacDonald et al., 2009).

1.5 Objectives

There are two main objectives in this thesis. The first objective is to validate our new USP4 antibody and use it to determine the expression levels of USP4 within mouse tissues in order to assess which areas of the organism (eventually leading to levels in the human body) may be affected by increased/decreased levels of USP4 as well as its mutations. The second objective is to better understand the role and regulation of USP4 within the cell.

1.6 Hypotheses

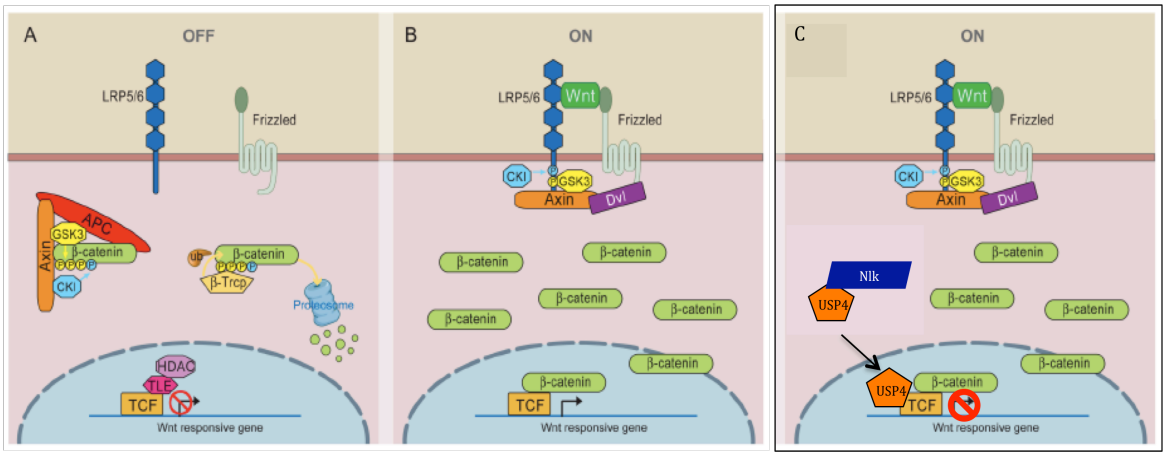
Based on the objectives stated above, there are three hypotheses generated.

1. Protein expression of USP4 will not differ significantly across tissue types, but as the organism ages, expression of USP4 may change.
2. Dysregulation of USP4 through the effects of canonical Wnt signaling plays a role in tumourigenesis.
3. USP4 activity may be regulated by phosphorylation.

1.7 Significance

This research is significant in that it intends to more thoroughly explain the biological relevance of USP4 in the cell and organism. There have been previous findings of interacting

Figure 3: Schematic representation of canonical Wnt signaling. In the absence of Wnt, cytoplasmic β-catenin is constantly being degraded by the destruction complex, consisting of the tumour suppressor proteins Axin, and APC and the two kinases, CK1 and GSK3α/β. Phosphorylation of β-catenin by these proteins targets it to the proteasome for degradation by E3 ligase β-Trcp, preventing it from translocating into the nucleus and activating transcription of the Wnt target genes. Without translocation of β-catenin in to the nucleus, TCF complexes with Groucho/Grg/TLE proteins, inhibiting transcription of Wnt responsive genes. In the presence of Wnt, the pathway is activated. The Wnt binds to the transmembrane receptor Frizzled and its co-receptor LRP6 (or LRP5), forming a complex that recruits Dishevelled (Dvl), resulting in the phosphorylation of LRP6 by glycogen synthase kinase 3β (GSK3β) and caseine kinase 1-γ (CK1γ). Phosphorylation of LRP6 recruits Axin, inhibiting Axin-mediated β-catenin phosphorylation, stabilizing β-catenin, leading to its accumulation and localization towards the nucleus to form complexes with transcription factors TCF and LEF and promote Wnt target gene expression (MacDonald et al., 2009). Also shown in the role of USP4 in inhibiting Wnt signaling prior to this study. With Wnt signaling turned 'on' USP4 interact with Nemo-like kinase (Nlk), resulting in its translocation to the nucleus. Upon entering the nucleus, USP4 interacts with TCF4, preventing the activation of Wnt responsive genes (Zhao et al., 2009). Figure adapted from (MacDonald et al., 2009).


A
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LRP5/6
Frizzled
APC
Axin
GSK3
β-catenin
CKI
β-catenin
β-Trcp
Proteasome
HDAC
TLE
TCF
Wnt responsive gene
B
ON
LRP5/6
Wnt
Frizzled
CKI
GSK3
Axin
Dvl
β-catenin
TCF
Wnt responsive gene
C
ON
LRP5/6
Wnt
Frizzled
CKI
GSK3
Axin
Dvl
Nlk
USP4
β-catenin
TCF
Wnt responsive gene

partners, possible substrates and pathways in which it is involved, however still very little is known of the biological role of USP4. In this work we uncover yet another dimension of USP4 in discovering one of its kinases, also involved in Wnt signaling, and that it has an effect on the levels of Beta-catenin within the cell. We also determine the expression levels of USP4 in both young and aged mouse tissues, which will help to determine the consequences of permutations in USP4 expression. This leads to a better understanding of its role within the cell and its implication in disease.

2. MATERIALS AND METHODS

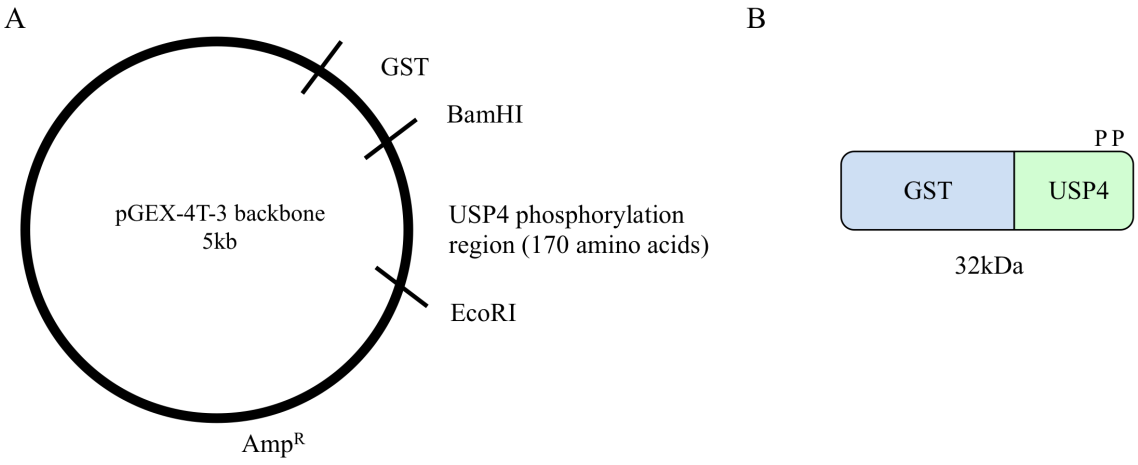
2.1 Cell culture and transfections

NIH3T3 cells were cultured at 37°C in 5% CO_2 in DMEM (Thermo Scientific) supplemented 10% fetal bovine serum. NIH3T3 cells were plated at 100 000 cells per well in 6-well plates the day prior to transient transfection. HEK293 cells were cultured at 37°C in 5% CO_2 in αMEM (Thermo Scientific) supplemented with 10% fetal bovine serum. Transfections were done using Lipofectamine 2000 (Invitrogen Canada Inc, Burlington, Ontario, Canada) or GeneJuice (Novagen, La Jolla, CA, USA) and 2-4ug DNA, as stated in specific procedure, according to manufacturer's protocol. Cells were incubated 48h post transfection, then analysed.

2.2 Generation of expression constructs

The expected region of USP4 to be phosphorylated was subcloned into expression vector pGEX-4T3 (GE Healthcare, Piscataway, NJ, USA) (figure 4). Primers were designed to incorporate a BamHI (NEB Biolabs, Pickering, Ontario, Canada) restriction site into the forward primer, and a EcoRI (NEB Biolabs, Pickering, Ontario, Canada) site into the reverse primer. GST-Phospho-USP4 Fwd primer: 5-CTAGGATCCATAAAACAGCCTTTG-3' GST-Phospho-USP4 Rev primer: 5'-ATTAGAATTCTCTCCCTGATCGTCCTC-3'. PCR performed using Platinum Taq DNA Polymerase (Invitrogen Canada Inc, Burlington, Ontario, Canada) using an already constructed FLAG-tagged wt-USP4 expression vector. The PCR product and the pGEX-4T3 backbone were double digested with BamHI and EcoRI with BSA at 37°C for 1 hour and ligated using T4 DNA ligase (NEB Biolabs, Pickering, Ontario, Canada)

Figure 4: GST-USP4 expression vector and peptide. A) Vector map of phosphorylated region of USP4 in pGEX-4T-3 backbone. This vector incorporates a GST tag to the N-terminal end of USP4, is ampicillin resistant, and has high expression upon IPTG induction. B) Schematic of the small GST-USP4 phosphorylated region peptide. The two P's show that the phosphorylated serines are towards the end of the peptide.


A
GST
BamHI
pGEX-4T-3 backbone
5kb
USP4 phosphorylation
region (170 amino acids)
EcoRI
Amp^R
B
P P
GST
USP4
32kDa

overnight at room temperature. An expression construct containing a myc-tagged USP4 with a mutation converting the cysteine residue present at the cataylytic site to an alanine residue (C311A) was already available. In order to FLAG tag this mutant version of USP4, the myc-tagged C311A USP4 plasmid as well as the WT FLAG-USP4 plamid were digested with BsrG1 and Nhe1, which cut on either side of the active site. The small fragment from the C311A USP4 digest was then ligated into the large fragment from the WT FLAG-USP4 digest using T4 DNA ligase (NEB Biolabs, Pickering, Ontario, Canada) overnight at room temperature. The expression constructs WT FLAG tagged USP4, P-Mut FLAG tagged USP4 (S675/680A) were previously made by other students in the lab.

2.3 Protein extraction

Cells were lysed using a protein lysis buffer (20mM Tris-HCl pH 7.5, 150nM NaCl, 1% Nonidet P-40, 0.5mM EDTA, 5% glycerol); tissues were homogenized then lysed with a lysis buffer containing 50mM Tris-HCl, 1mM EDTA, 250mM sucrose, 25mM KCl, 5mM $MgCl_2$, 2mM DTT. Both were supplemented with 2mM sodium orthovanadate ($NaVO_4$), 2mM sodium fluoride (NaF), 2mM sodium pyrophosphate (NaPPi), 200ug/mL phenylmethylsulfonyl fluoride (PMSF) and 1X protease inhibitor cocktail (Roche Applied Science, Laval, Quebec, Canada) 48h post transfection. Samples were sonicated 3 times for 10 seconds each, placed on each between sonications. Lysates were centrifuged at 14 000rpm at 4°C for 20 minutes, and supernatants transferred to new tube. The protein concentration was determined using Bradford protein assay (Bio-Rad Laboratories Canada Inc, Mississauga, Ontario, Canada).

2.4 Western blot analysis

Lysates were diluted with SDS sample buffer (0.25M Tris HCl, 8% SDS, 40% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) and heated to 100°C for 5 minutes. Protein samples (40ug/lane of cell lysates or 50ug/lane tissue lysates) were resolved by on a 4-10% Bis-Tris gel by SDS-PAGE (Invitrogen Canada Inc, Burlington, Ontario, Canada) and electroblotted onto hydrobond C nitrocellulose membrane (Amersham Pharmacia Biotechnologies, Baie D'Urfée, Quebec, Canada). Membranes were stained using Ponceau S (Sigma-Aldrich Co, St-Louis, Missouri, USA) to verify for complete protein transfer. Membranes were incubated 1h in 5% skim milk in TBST (10mM Tris-HCl pH 7.5, 150mM NaCl and 1% Tween-20), then incubated in respective primary antibody diluted in 5% skim milk in TBST and incubated overnight at 4°C or at room temperature for 1h. Membranes were then wash 3 times for 10 minutes in TBST, then incubated for 1h at room temperature in corresponding secondary antibody diluted in 5% skim milk in TBST. Again the membrane was washed 3 times for 10 minutes in TBST and proteins were visualized using horseradish peroxidase ECL kit from Thermo Scientific.

2.5 Immunoprecipitation

Cells were transiently transfected with WT or P-mut or C311A FLAG USP4 using Lipofectamine 2000 (Invitrogen Canada Inc, Burlington, Ontario, Canada). FLAG immunoprecipitations were performed using ANTI-FLAG M2-Agarose beads (Sigma-Aldrich Co, St-Louis, Missouri, USA). 40uL of FLAG beads were pipeted into tube and centrifuged to remove supernatant. Beads were washed three times with 400uL lysis buffer used to lyse cells. Total protein from cell lysates were quantitated using Bradford protein assay (Bio-Rad

Laboratories Canada Inc, Mississauga, Ontario, Canada), and 750ug protein was added to the beads. Slurry was incubated on rotator at 4°C for 2 hours, and then spun down to remove supernatant. Beads were washed 3 times with lysis buffer. FLAG-tagged protein was removed from beads by added 25uL 3X FLAG Peptide (Sigma-Aldrich Co, St-Louis, Missouri, USA), and incubated at 65°C with shaking for 10 minutes. Slurry was spun down and supernatant was collected for analysis.

β-catenin and GRK2 immunoprecipitations were performed using 750μg of protein, incubated with 40μL GammaBind G Sepharose (GE Healthcare, Piscataway, NJ, USA) bead slurry (previously washed three times with lysis buffer) overnight at 4°C with shaking. The following day, antibody was added to a 1:200 dilution and incubated for 2 hours at 4°C with shaking. Beads were then washed three more times with lysis buffer, then proteins were eluted by adding 20μL of 3X SDS sample buffer (187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue).

2.6 Antibodies

The companies from which the antibodies were obtained and the dilutions of the antibodies for western blot analysis and immunoprecipitations are summarized in Table 1.

Table 1: Antibody companies and dilutions

		Dilution	
Antibody	Company	WB	IP
USP4	Aves Labs Inc	1:1500	1:100
USP15	Aves Labs Inc	1:750	-
Beta-catenin	Cell Signaling	1:1000	1:200
GRK2	Millipore	1:1000	1:100
Actin	Sigma	1:8000	-

2.7 GST protein purification

GST-phospho-USP4 expression plasmid was transformed according to standard procedures into competent E. coli cells, plated and grown on LB-Amp plates overnight at 37°C. Colonies were picked the next day and grown up overnight on a shaker at 37°C in 3mL LB-Amp with chloramphenical (Sigma-Aldrich Co., St-Louis, Missouri, USA). Next day, 0.5mL of overnight culture was diluted into 4mL LB-Amp and grown up 1 hour at 37°C on shaker. After 1 hour of growth, 1mM IPTG (Sigma-Aldrich Co., St-Louis, Missouri, USA) was added to sample and grown until OD600 = 1.5-3.0. Samples were then centrifuged at 6000g for 15 minutes to pellet bacteria. GST fusion proteins were then purified using Pierce's Bacterial GST Fusion Protein Purification Kit (Thermo Fisher Scientific, Rockford, IL, USA) as per manufacturer's protocol. Purified protein was then dialyzed using SnakeSkin Dialysis tubing and clips (Thermo Fisher Scientific, Rockford, IL, USA) to change buffer to 20mM Tris pH 7.5, 50mM NaCl.

2.8 USP4 and GRK2 knockdown

USP4 knockdown

NIH3T3 cells were plated onto 6-well dishes at 50 000 cells/well. The following day, cells were left untransfected or transfected using GeneJuice (Novagen, La Jolla, CA, USA). Cells were transfected with 3ug of MISSION TurboGFP control (SHC003) or 3ug of MISSION shRNA Plasmid DNA clones (NM_011678): TRCN0000295779 (clone A), TRCN0000030739 (clone B), TRCN0000030740 (clone C), TRCN0000030741 (clone D), TRCN0000030743 (clone E) (Sigma-Aldrich Co., St-Louis, Missouri, USA). All clones had differing shRNA

sequences, targeting different portions of USP4. Cells were collected 48 hours post-transfection for protein analysis.

GRK2 knockdown

HEK293 cells were plated onto 6-well dishes at 100 000 cells/well. The following day, cells were left untransfected or transfected using Oligofectamine Reagent (Invitrogen Canada Inc, Burlington, Ontario, Canada) according to manufacturer's protocol. Cells were transfected with 200nM oligonucleotide siRNA: GRK2 siRNA1 (GCA AGA AAG CCA AGA ACA AUU), GRK2 siRNA2 (AAG AAG UAC GAG AAG CUG GAG UU), or nonsilencing control (AAU UCU CCG AAC GUG UCA CGU UU) (Thermo Scientific), as previously determined to work by other laboratories (Kim et al., 2005; Namkung et al., 2009). 24 hours later, cells were left alone, or transfected with WT, Pmut or C311A FLAG-USP4 using GeneJuice transfection reagent (Novagen, La Jolla, CA, USA). Cells were collected 24h post transfection (48h post GRK2 knockdown) for analysis.

2.9 Flow cytometry

NIH3T3 cells were plated 100 000 cells/well in a 6-well dish. The following day, 3ug of clone A USP4 shRNA (TRCN0000295779) or a non-target shRNA control vector (SHC002) (Sigma-Aldrich Co., St-Louis, Missouri, USA) was transfected using Lipofectamine 2000 (Invitrogen Canada Inc, Burlington, Ontario, Canada) according to manufacturers protocol. After 48h incubation, cells were trypsinized and all six wells were pooled then pelleted and washed by resuspending in PBS. Cells were again pelleted, then resuspended in 70% ethanol and stored at -20°C until staining. Cells were pelleted by centrifugation at 2000rpm for 5 minutes at 4°C. Supernatent (ethanol) was aspirated, and pellet was resuspended in PBS. Cells

were centrifuged again at 2000rpm for 5 minutes at 4°C, supernatant was aspirated and cells were resuspended in 1mL propidium iodide staining buffer (50ug/mL PI in PBS + 40ug/mL Rnase A) and incubated for 30 minutes 30 minutes at room temperature in the dark. DNA content was analyzed using flow cytometry with a BD LSR flow cytometer (Becton Dickinson, San Jose, CA, USA). Data was acquired using Cell Quest software (Becton Dickinson, San Jose, CA, USA), and results were analyzed using Mod Fit LT software (Verity Software House, Inc., Sophsam, ME).

2.10 USP4 deubiquitinating activity assay

HEK293 lysates from cells with GRK2 knocked down in combination with overexpression of WT or Pmut Usp4, and lysates from cells with either WT, Pmut, or C311A Usp4 overexpression were immunoprecipitated using ANTI-FLAG M2-Agarose beads (Sigma-Aldrich Co, St-Louis, Missouri, USA). 40μL of beads were washed three times in Frack's lysis buffer, then immunoprecipitated for 2 hours with 600μg total protein at 4°C on a rotator. Beads were then washed three times in Frack's lysis buffer. As a negative control, 10mM NEM (a thiol-blocking reagent which deactivates the active cysteine in deubiquitinating enzymes) (Stipanuk et al., 2004) was added to a sample prior to immunoprecipitation. Flag-tagged protein was not eluted from the beads, but instead transferred to a 96-well sample plate. Deubiquitinating activity was measured using DUB-Glo™ Protease Assay (Promega Corporation, Madison, WI, USA). 50μL of DUB-Glo reagent was added into each sample well and incubated for 30 minutes on a shaker at room temperature. Luminescence was then read with a SynergyMx Monochromator-Based Multi-Mode Microplate Reader (Biotek Instruments,

Inc., Winooski, VT, USA) and data collected by Gen5 Data Analysis Software (Biotek Instruments, Inc., Winooski, VT, USA).

3. RESULTS

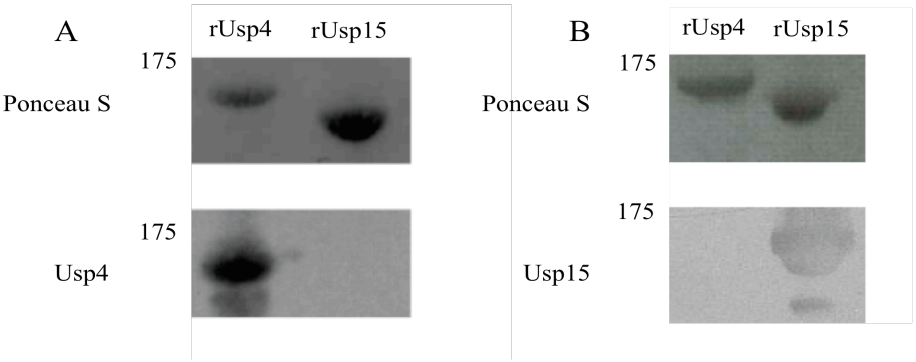
3.1 Specificity of USP4 and USP15 antibodies

USP4 and USP15 have been found to have very high sequence similarity (76%), which can pose a problem when using an antibody to detect the protein (Angelats et al., 2003; Baker et al., 1999). To remedy this, we contracted the production of polyclonal chicken IgY antibodies specific to USP4 and USP15 by Aves Labs Inc. (Tigard, OR, USA). The USP4 antibody epitope is specific to amino acids $_{664}$MDHQEEGKEQ$_{673}$ on USP4, which is an unconserved region in USP15. The USP15 antibody epitope is DEDSNDNDNDLENENC, which is also unconserved in USP4. To verify the specificity of these antibodies, recombinant USP4 and USP15 (LifeSensors, Malvern, PA, USA) were run on SDS-PAGE for western blot, probing with these antibodies. The western blot (Figure 5) shows that the USP4 recombinant protein is only picked up by the USP4 antibody, and not the USP15 antibody, and vice versa. This confirms that the newly designed antibodies are specific to USP4 and USP15, respectively.

3.2 Comparative Protein Expression Analysis

Previous work has shown that expression of USP4 at the RNA level is uniformly expressed in the mouse liver, kidney, brain, lung and testes (Gupta et al., 1994). Expression of USP4 at the protein level, however, had not yet been established. With our new, specific antibody we have been able to do so. Harvesting the mouse tissues and extracting the protein as described, we ran the tissue lysates on a SDS-PAGE for Western blot analysis. We began by looking at USP4 expression in tissues from a three month old FVB/N mouse. Figure 6 shows that there seem to be bands of unexpected sizes picked up with the USP4 antibody. There is a

Figure 5: Specificity of Usp4 and Usp15 antibodies. The custom made Usp4 and Usp15 antibodies are specific to themselves only, and do not cross-react with each other. A) Western blot analysis of recombinant Usp4 (rUsp4) and recombinant Usp15 (rUsp15) shows that there is recombinant protein in both lanes, but that the Usp4 antibody picks up only rUsp4 and not rUsp15. Conversely, B) shows by Western blot analysis that the Usp15 antibody picks up only rUsp15 and not rUSP4.


A
rUsp4
rUsp15
175
Ponceau S
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Usp4
B
rUsp4
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Ponceau S
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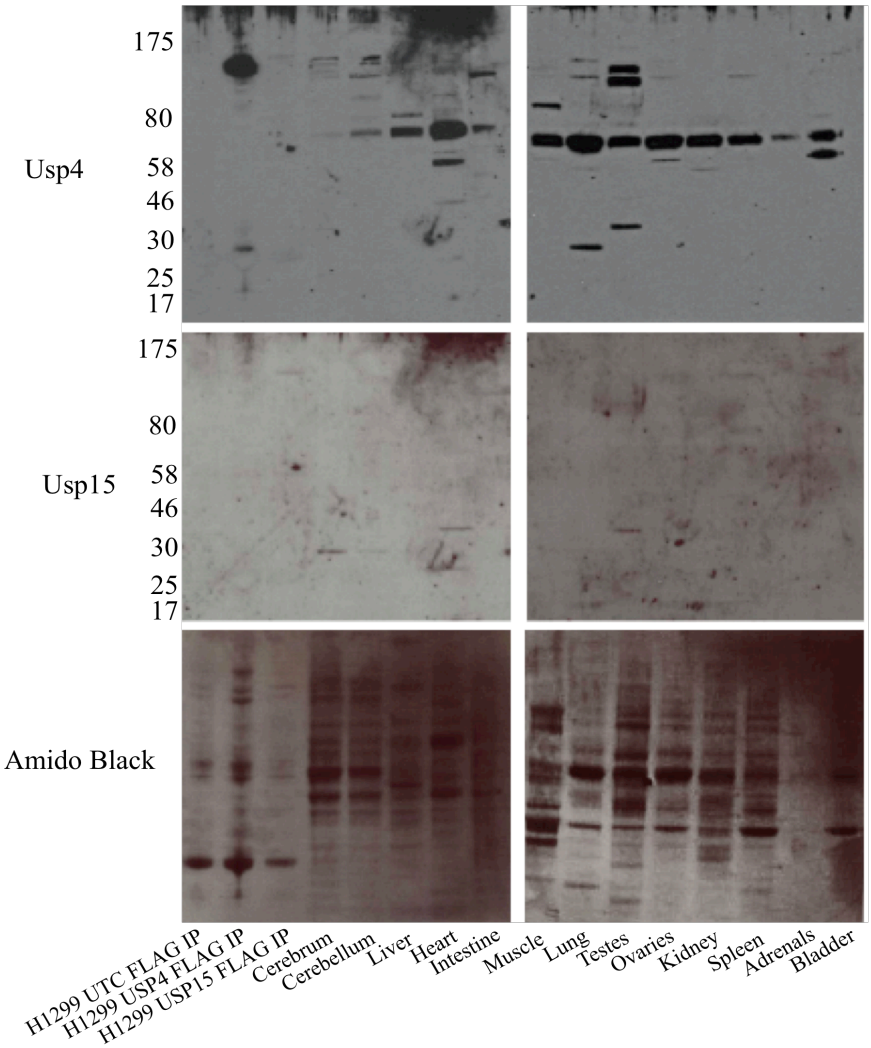
Usp15

Figure 6: Comparison of Usp4 and Usp15 protein expression in 3 month old FVB/N mice. Tissues from 3 month FVB/N mice were harvested and lysed then run on SDS-PAGE for western blot analysis, probing for Usp4 and Usp15. Usp4 has much higher expression than Usp15 in these mouse tissues; there are bands of unexpected sizes picked up with the USP4 antibody. There is a band present in most of the tissues at a molecular weight between 58 and 80kDa. Closer to the 108kDa expected size for USP4, we see three to four different sized isoforms in the brain samples (cerebrum and cerebellum). In the muscles, kidney and adrenals we do not see a band at the expected size, and both in the lung and the testes we see two differently sized isoforms, much more prominent in the testes than the lung. There is very little protein picked up in these samples with the Usp15 antibody, and any bands that are visible tend to be at much lower molecular weights than the predicted 112kDa. The membranes were stained with Amido black to show total protein and equal loading of wells.


Usp4
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Lung
Testes
Ovaries
Kidney
Spleen
Adrenals
Bladder

band present in most of the tissues at a molecular weight between 58 and 80kDa. Closer to the 108kDa expected size for USP4, we see three to four different sized isoforms in the brain samples (cerebrum and cerebellum). In the muscles, kidney and adrenals we do not see a band at the expected size, and both in the lung and the testes we see two differently sized isoforms, much more prominent in the testes than the lung. Also in Figure 6 we see that USP15 expression is much lower than that of USP4 in mouse tissues. There is very little protein picked up in these samples from the three months FVB/N mouse tissues, and any bands that are visible tend to be at much lower molecular weights than the predicted 112kDa. The membranes were stained with Amido black to show total protein and equal loading of wells. To learn about how USP4 expression changes as the organism ages, we collected tissues from a two year old FVB/N mouse. After protein extraction and running on SDS-PAGE for Western blot analysis, we see that again there are several different-sized bands picked up with the USP4 antibody, with a prominent band between 58 and 80kDa (Figure 7). In the two year old mouse, USP4 expression is approximately unchanged as compared to the three month old mouse, showing faint expression across most tissue types. However there is a strong difference when comparing USP4 expression in the testes. Whereas the three month old FVB/N mouse shows high USP4 expression with two different sized isoforms, the two year old mouse shows much less expression of the protein, with one prominent band at the expected size of 108kDa. USP15 expression in these tissues again is very low, with no bands picked up in the 112kDa range, only smaller isoforms of approximately 30kDa in the brain samples and liver. Finally, we looked at expression of USP4 and USP15 in a three month old C57 mouse (Figure 8). Expression levels of USP4 were comparable to those found in the young FVB/N mouse. Multiple isoforms were present in the brain samples, and most tissues had at least low levels of

Figure 7: Comparison of Usp4 and Usp15 protein expression in 2 year old FVB/N mice. Tissues from 2 year old FVB/N mice were harvested and lysed then run on SDS-PAGE for western blot analysis, probing for Usp4 and Usp15. USP4 expression is approximately unchanged as compared to the three month old mouse, showing faint expression across most tissue types with a band present in most of the tissues at a molecular weight between 58 and 80kDa. Contrary to what we have seen in the three month old FVB/N mouse, the two year old mouse shows much less expression of Usp4 in the testes, with one prominent band at the expected size of 108kDa. USP15 expression in these tissues again is very low, with no bands picked up in the 112kDa range, only smaller isoforms of approximately 30kDa in the brain samples and liver. Amido black shows even loading of protein within the wells.

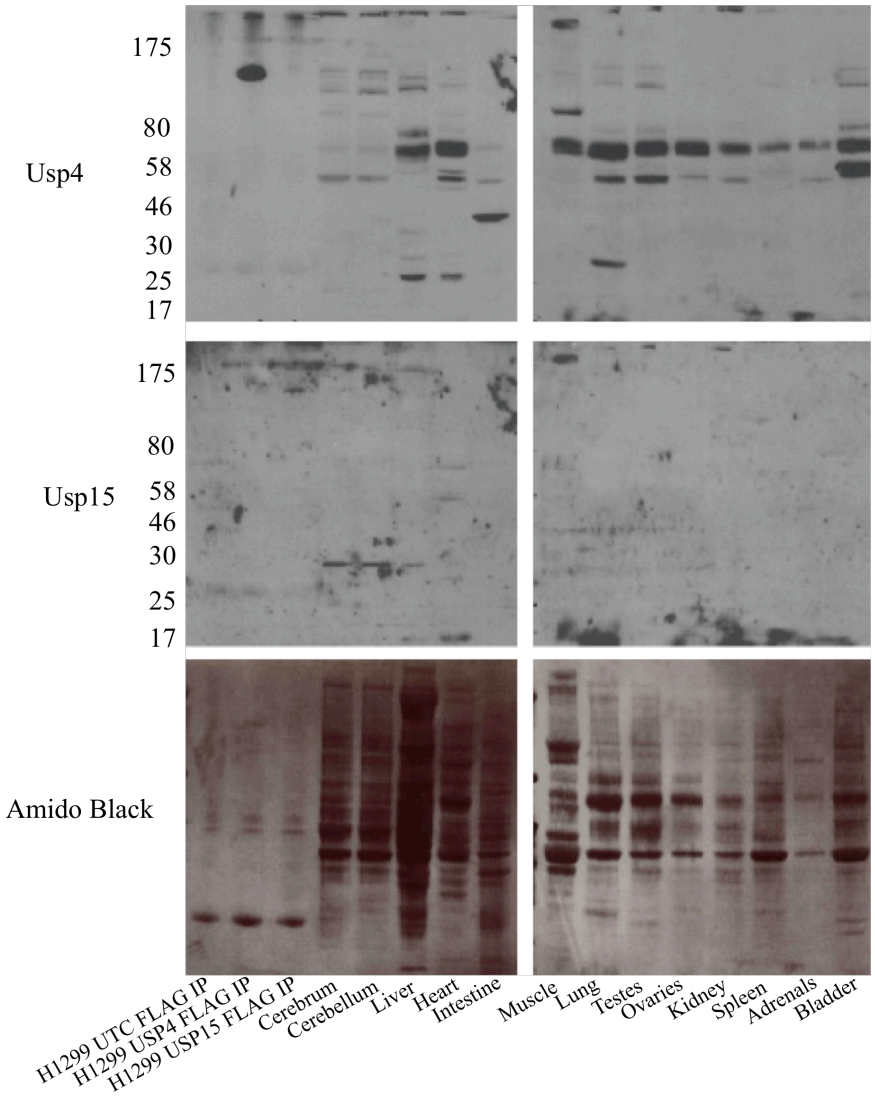
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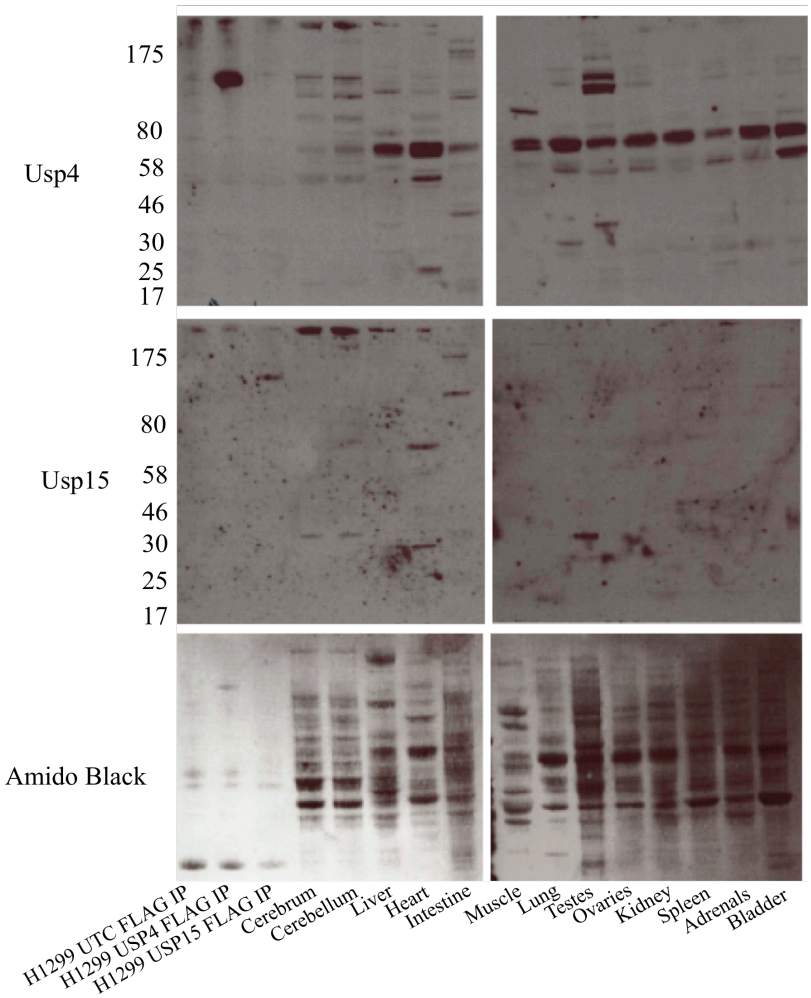
Figure 8: Comparison of Usp4 and Usp15 protein expression in 3 month old C57 mice. Tissues from 3 month C57mice were harvested and lysed then run on SDS-PAGE for western blot analysis, probing for Usp4 and Usp15. Expression levels of USP4 were comparable to those found in the young FVB/N mouse. Multiple isoforms were present in the brain samples, and most tissues had at least low levels of expression. Again, we see the two different sized isoforms found in the testes. There was more USP15 protein expressed in this C57 mouse than in the FVB/N mouse, however, with bands being picked up at the expected size of 112kDa in the intestine, spleen, and bladder.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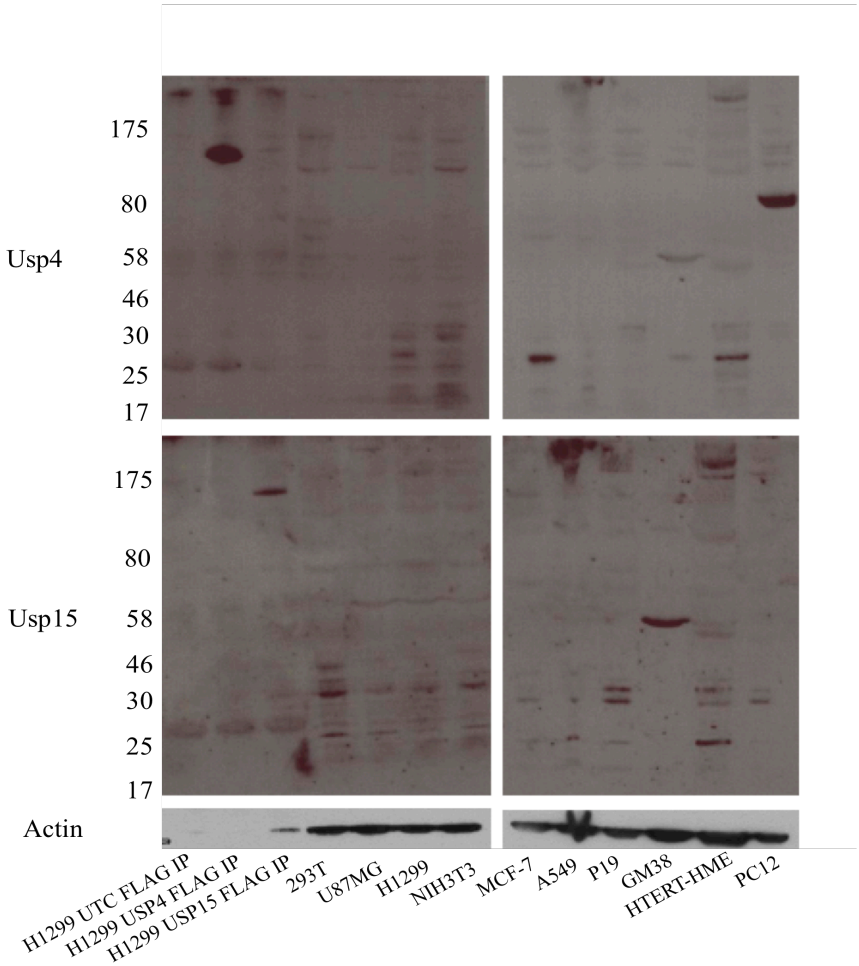
expression. Again, we see the two different sized isoforms found in the testes. There was more USP15 protein expressed in this C57 mouse than in the FVB/N mouse, however, with bands being picked up at the expected size of 112kDa in the intestine, spleen, and bladder. Figure 9 shows that low levels of USP4 at its expected molecular weight was found in each cell line, with slightly more expressed in NIH3T3 cells, which are immortalized mouse embryonic fibroblasts, and 293T cells, which are human embryonic kidney cells containing sheared adenovirus 5 DNA and expressing the SV40 Large T-antigen (Graham et al., 1977). There is also a very prominent band picked up at ~80kDa in the PC12 cell line.

3.3 Knockdown of USP4 using shRNA plasmid

One way to determine which bands from the mouse tissue and cell lysate samples are specific to USP4, and which are simply nonspecific binding of the antibody is to knock down the protein. We obtained from Sigma several clones of plasmid DNA expressing shRNA specific to different areas of USP4. Looking at the panel of cell lines studied for USP4 expression, we see that NIH3T3 cells have one of the highest amounts of USP4 expressed, and is a "normal" cell line – ie. not a cancer cell line, though it has been immortalized, so these cells were used for the knockdown (Figure 10A). Five different clones were used for the knock down, and although two of the clones were relatively good, clone A had virtually a complete knockdown, and thus this clone was used in all future experiments. We see that the only band that disappears with this knockdown is the band at the expected molecular weight of 108kDa, so when comparing USP4 expression between cell lines and tissues, we will look only at this molecular weight, and assume that the other bands are in fact not different isoforms, but simply nonspecific binding of the antibody. Figure 10B shows immunofluorescence of the positive

Figure 9: Comparison of Usp4 and Usp15 protein expression in various cell lines. Cells were grown to approximately 80% confluency then lysed in Frack's lysis buffer, and ran on SDS-PAGE for western blot analysis, probing for Usp4 and Usp15, with actin showing equal loading of all samples. Low levels of USP4 at its expected molecular weight was found in each cell line, with slightly more expressed in NIH3T3 cells, which are immortalized mouse embryonic fibroblasts, and 293T cells, which are human embryonic kidney cells containing sheared adenovirus 5 DNA and expressing the SV40 Large T-antigen (Graham et al., 1977). There is also a very prominent band picked up at ~80kDa in the PC12 cell line. This size of isoform has not been seen in any other sample, and is interesting because PC12 is a cell line derived from the rat adrenal medulla, and it has been found that USP4 levels are increased in adrenocortical tumours (Laurell et al., 2009).


Usp4
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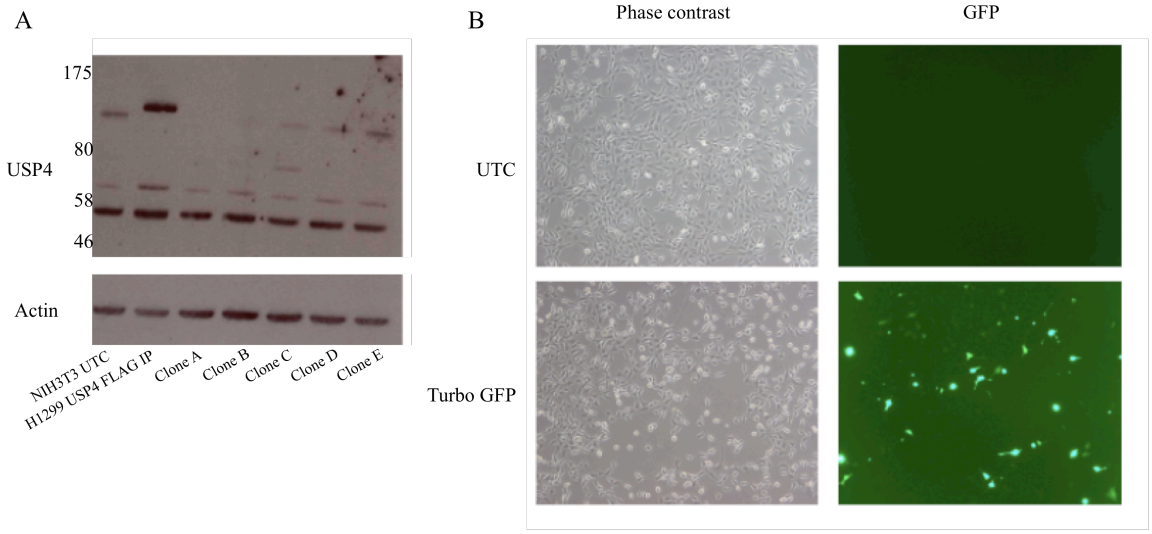
H1299 USP4 FLAG IP
H1299 USP15 FLAG IP
293T
U87MG
H1299
NIH3T3
MCF-7
A549
P19
GM38
HTERT-HME
PC12

Figure 10: Knockdown of Usp4 using shRNA in NIH3T3 cells. NIH3T3 cells were used to knock down Usp4 using MISSION shRNA plasmid DNA clones. A) Five different clones were used for the knock down, and although two of the clones were relatively good, clone A had virtually a complete knockdown, and thus this clone was used in all future experiments. We see that the only band that disappears with this knockdown is the band at the expected molecular weight of 108kDa. B) Transfection efficiency of this plasmid has been shown by immunofluorescence, as the regular GFP antibody does not pick up TurboGFP. Cells were left untransfected or transfected with this TurboGFP positive control, showing good transfection efficiency of the plasmid into the cells.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58
46
USP4
Actin
NIH3T3 UTC
H1299 USP4 FLAG IP
Clone A
Clone B
Clone C
Clone D
Clone E
B
Phase contrast
GFP
UTC
Turbo GFP

control for the transfection of the plasmid expressing the shRNA. This is transfection of the same plasmid, containing instead of the USP4 shRNA, a TurboGFP sequence. Because the regular GFP antibody does not pick up TurboGFP, we have used immunofluorescence to confirm visually the transfection efficiency of the plasmid.

3.4 Analysis of USP4 knockdown on cell cycle

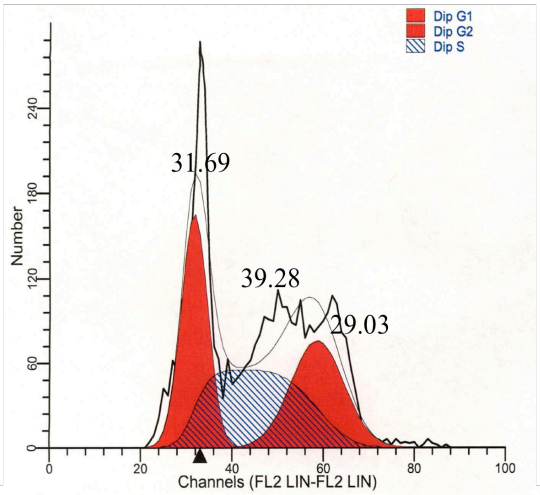
As has been previously found by Song et al., depletion of USP4 leads to impaired cell cycle progression and splicing (Song et al., 2010). To delve deeper into the role of USP4 and its role in cell cycle control, we performed cell cycle flow cytometry on cell which have had USP4 knocked down by shRNA. Figure 11A shows our flow cytometry result with normal, untransfected NIH3T3 cells. Both characteristic G1 and G2 peaks are clearly visible, with S phase shown in between. These show that the cells were healthy and growing at the time of the experiment. Figure 11B shows that there is no change in the cell cycle when transfected with a non-targeting control shRNA, as predicted. On the other hand, Figure 11C shows that the NIH3T3 cells with USP4 knocked down by shRNA do have the characteristic G1 peak, but the G2 peak is gone.

3.5 Effect of Usp4 on Beta-catenin stability

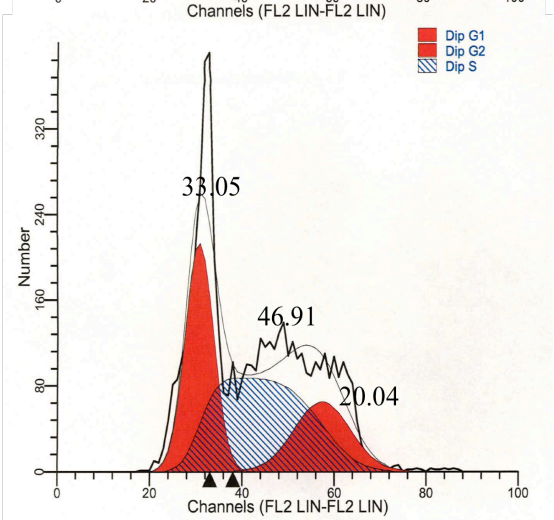
It was recently shown that USP4 is implicated within the canonical Wnt signaling pathway, and that its depletion results in an upregulation of the pathway (Zhao et al., 2009). The authors also speculate that USP4 is acting on the pathway downstream from the destruction complex and stabilization of Beta-catenin. On the other hand, in Figure 12 we demonstrate that knock down of USP4 in NIH3T3 cells actually causes a degradation of Beta-catenin.

Figure 11: Effect of Usp4 knockdown on cell cycle by flow cytometry. Analysis of the effect of Usp4 knockdown on the cell cycle in NIH3T3 cells comparing untransfected cells with a nontargeting control and Usp4 shRNA using propidium iodide staining. A) Normal, untransfected NIH3T3 cells have both characteristic G1 and G2 peaks clearly visible, with S phase shown in between. These show that the cells were healthy and growing at the time of the experiment. B) shows that there is no change in the cell cycle when transfected with a non-targeting control shRNA, as predicted. On the other hand, (C) shows that the NIH3T3 cells with Usp4 knocked down by shRNA do have the characteristic G1 peak, but the G2 peak is nonexistent, indicating problems with the synthesis stage of the cell cycle.

A


Dip G1
Dip G2
Dip S
31.69
39.28
29.03
Number
Channels (FL2 LIN-FL2 LIN)

B


Dip G1
Dip G2
Dip S
33.05
46.91
20.04
Number
Channels (FL2 LIN-FL2 LIN)

C

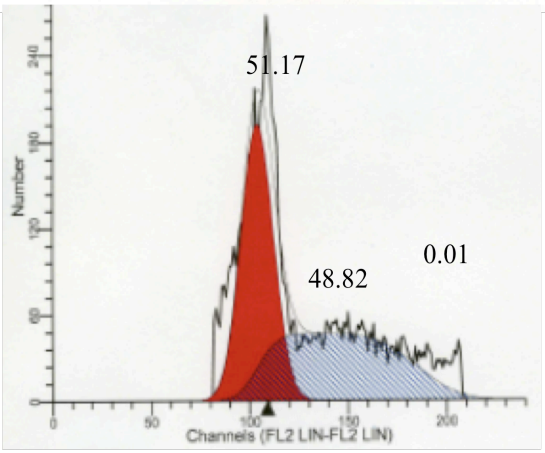
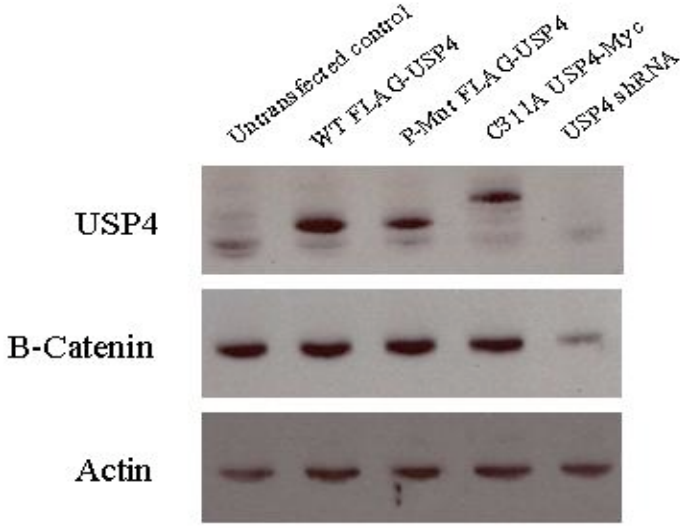

51.17
48.82
0.01
Number
Channels (FL2 LIN-FL2 LIN)

Figure 12: Usp4 knockdown negatively affects B-catenin stability. NIH3T3 cells were cultured and transfected with constructs to overexpress Usp4, as shown, or to knock down Usp4. Western blot analysis shows that B-catenin stability is maintained when cells have been overexpressed with WT, phosphorylation mutant and the catalytic mutant (C311A) Usp4. However with knockdown of Usp4 by shRNA, we see that the level of B-catenin expressed within the cell decreases dramatically. Actin blot shows equal loading of all wells.


Untransfected control
WT FLAG-USP4
P-Mut FLAG-USP4
C311A USP4-Myc
USP4 shRNA
USP4
B-Catenin
Actin

Overexpression of the wildtype, phosphorylation mutant and catalytic mutant has no effect on Beta-catenin levels.

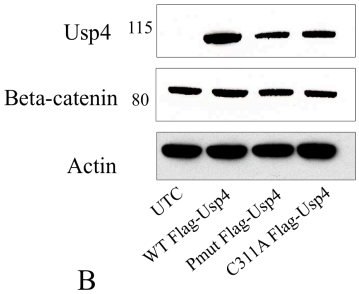
Given that we have seen that USP4 may influence B-catenin stability (as shown in Figure 12), we decided to look at whether or not the two proteins interact long enough to co-immunoprecipitate. Figure 13A shows the levels of both the overexpressed USP4 and endogenous Beta-catenin in the sample before immunoprecipitation. Figure 13B shows that neither protein co-immunoprecipitates with the other. When pulling down USP4 with either the FLAG peptide or the USP4 antibody with sepharose beads, Beta-catenin is not detected, and conversely, when Beta-catenin is pulled down using its antibody and sepharose beads, USP4 is also not detected.

3.6 USP4 Kinase Screen

Unpublished mass spectrometry data from the Gray lab shows that USP4 is phosphorylated at serines 675 and 680. It is plausible that the identification of the kinase responsible for USP4 phosphorylation may shed a light as to which pathway USP4 features in, and how USP4 is itself regulated. For the purpose of screening a library of mammalian kinases a plasmid was constructed that encoded a region of USP4 containing the previously identified serines fused to glutathione S transferase (Figure 4). The fusion peptide was expressed in E. coli by IPTG induction and isolated by glutathione affinity beads (Figure 14). Table 2 shows the summarized results of a kinase screen performed on this fusion peptide in collaboration with the laboratory of Dr. Rob Screaton (see appendix for the full results). Listed in the table is the name of the kinase that had a positive hit, as well as the USP4:MBP ratio (on a scale of high, medium, low) which indicates specificity of the kinase for USP4. A high USP4:MBP

Figure 13: Usp4 and B-catenin do not co-immunoprecipitate. Cell lysates overexpression Usp4 and immunoprecipitated with either Flag, Usp4 or B-catenin show that Usp4 and B-catenin do not co-immunoprecipitate, as there is no detection of the other protein when run on SDS-PAGE for western blot analysis. A) shows the levels of protein in the samples before immunoprecipitation, and B) shows that when samples are immunoprecipitated with B-catenin and sepharose beads, Usp4 is not pulled down with it, and vice versa; when Usp4 is immunoprecipitated using either Flag beads or the Usp4 antibody with sepharose beads, no B-catenin is detected in these samples.

A


Usp4
115
Beta-catenin
80
Actin
UTC
WT Flag-Usp4
Pmut Flag-Usp4
C311A Flag-Usp4

B

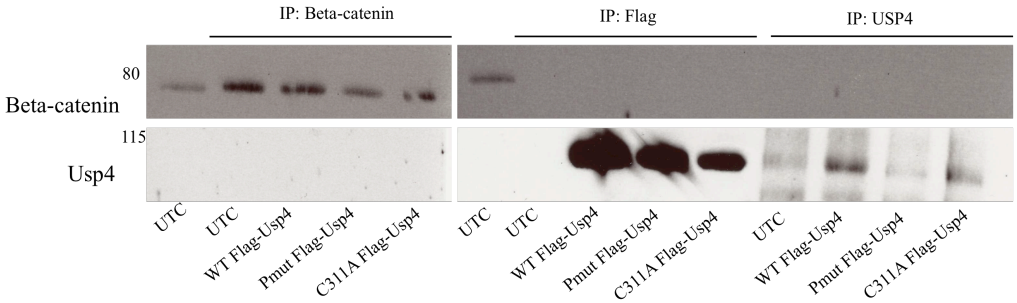

IP: Beta-catenin
IP: Flag
IP: USP4
80
Beta-catenin
115
Usp4
UTC
UTC
WT Flag-Usp4
Pmut Flag-Usp4
C311A Flag-Usp4
UTC
UTC
WT Flag-Usp4
Pmut Flag-Usp4
C311A Flag-Usp4
UTC
WT Flag-Usp4
Pmut Flag-Usp4
C311A Flag-Usp4

Figure 14: GST-USP4 peptide expression and isolation. A) The GST-USP4 peptide was overexpressed in E.coli cells using 1mM IPTG induction. The resulting protein has a molecular weight of 32kDa. B) The GST-USP4 peptide was purified using a GST protein purification kit, and run on SDS-PAGE and Coomassie stained to look for any degradation. 1, 2, and 5ug of GST-USP4 was run alongside BSA as a control, and we see that the protein is perfectly intact, with no degradation.

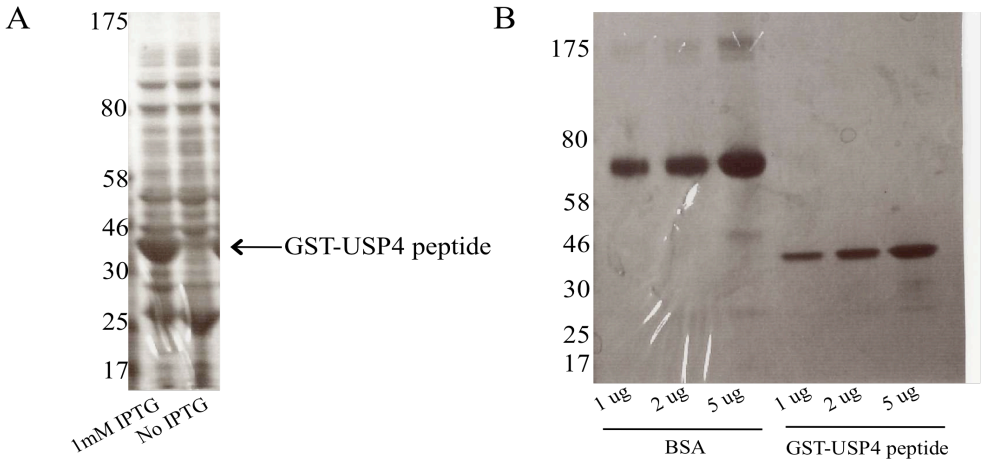

A
175
80
58
46
30
25
17
GST-USP4 peptide
1mM IPTG
No IPTG
B
175
80
58
46
30
25
17
1 ug
2 ug
5 ug
1 ug
2 ug
5 ug
BSA
GST-USP4 peptide

Table 2: USP4 primary kinase screen results. A GST-Usp4 fusion peptide was generated by attaching the region of Usp4 predicted to be phosphorylated (which includes the two serines at positions 675 and 680, as well as several other serines in the vicinity, as described in methods), making a peptide-GST fusion of 33kDa. This peptide was purified and sent for the kinase screen and the following results were obtained. Shown here are all of the positive hits, along with their USP4:MBP ratio on a scale of high, medium, low) which indicates specificity of the kinase for Usp4. A high Usp4:MBP ratio indicates that the kinase is more specific to Usp4 than one with a low ratio, and that it is more likely to be a 'real' hit rather than a forced hit due to the *in vitro* nature of the assay.

Gene	USP4:MBP
ADRBK1	moderate
BMPR1A/ ALK-3	moderate
CSNK1D	low
CSNK1E	low
CSNK1G2	low
GRK1	high
GRK6	high
MAPRK14	low
MAP3K3	low
MARK4	moderate
MGC42105	low
NEK4	low
NEK9	low
PBK	
PHKG1	low
PKN2/PRKCL2	low
PKN3	low
PLK4	moderate
RPS6KA6	low
SYK	low
TTBK1	moderate
ULK1	low
YSK4	high

ratio indicates that the kinase is more specific to USP4 than one with a low ratio, and that it is more likely to be a 'real' hit rather than a forced hit due to the *in vitro* nature of the assay.

Upon receiving the results from the primary kinase screen, we provided the Screaton laboratory with more of the recombinant protein fragment for a secondary screen, focusing on the positive hits we received in the primary screen, excluding the kinases which tend to target lots of substrates such as NEK4 and NEK9, GRK1 and GRK6 (Rob Screaton, personal communication). The results of the secondary screen are summarized in Table 3 and the full results including the gels are within the appendix. Out of 23 kinases screened, there were four that showed positive hits; ADRBK1 (GRK2), MARK4, and TTBK1. These results show that the most positive hit we have for the phosphorylation of USP4 is by G protein-coupled receptor kinase 2 (GRK2).

3.7 Effect of GRK2 knockdown on USP4 enzymatic activity

To determine of the effect of USP4 phosphorylation by GRK2, we first began by knocking down GRK2 using siRNA in HEK293 cells. Figure 15 shows efficient knockdown using both siRNA1 and siRNA2 when the cell's only perturbation is the knockdown. However, upon perturbing the cells 24h post knockdown by overexpression of either WT or Pmut USP4, the knockdown efficiency decreases. Using an assay kit available from Promega, which uses a luminescence signal produced by the cleavage of aminoluciferin from the C-terminal pentapeptide of ubiquitin (Promega Corporation, Madison, WI, USA), we have determined the activity of various forms of Usp4 with and without the knockdown of GRK2. Cells were manipulated as stated in the materials and methods section and lysed. Figure 16A shows that the efficiency of the Flag IP on all of the samples were approximately even, so any fluctuations

Table 3: USP4 secondary kinase screen results. A secondary kinase screen was performed after obtaining the results of the primary screen. The secondary screen was done by redoing the primary screen, focusing on only the positive hits from the primary screen to look for repeats. Shown here are the hits for this second screen. A dash in USP4:MBP ratio shows that there was not a hit this second time. ADRBK1 (GRK2) has the greatest specificity ratio, suggesting that it is the most likely kinase to be phosphorylating this region of Usp4.

Gene	USP4:MBP
MAP3K14	-
YSK4	-
NEK4	-
PKN3	-
PLK4	-
CSNK1G2	-
SYK	-
CSNK1D	-
CSNK1E	-
GRK6	-
ADRBK1 (GRK2)	high
MAP3K3	-
PHGK1	-
TOPK	-
NEK9	-
MGC42105	-
RPS6KA6	-
MARK4	low
PRKCL2	-
ULK1	-
GRK1	-
TTBK1	low
ALK3	-

Figure 15: Knockdown of GRK2 using siRNA in HEK293 cells. HEK293 cells were cultured and transfected with GRK2 siRNA for 48h to knockdown GRK2. 24h post knockdown, cells were either left alone, transfected with a pcDNA3 vector control, or with WT or Pmut Usp4. The knockdown is very efficient when cells are treated only with the siRNA (both siRNA 1 and 2 have about equal efficiency), but when the cells are again perturbed by overexpression 24h post knockdown, GRK2 levels increase, thus decreasing efficiency of the knockdown.

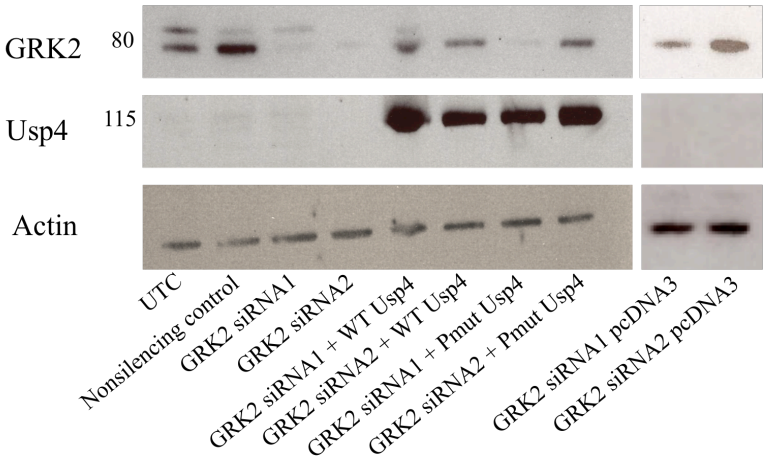
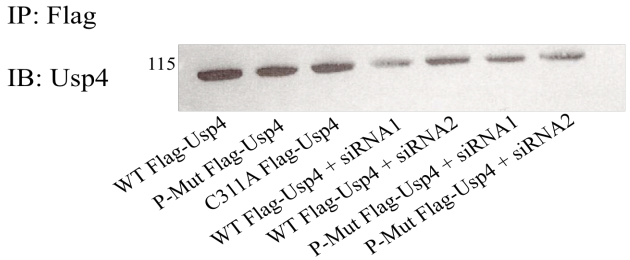
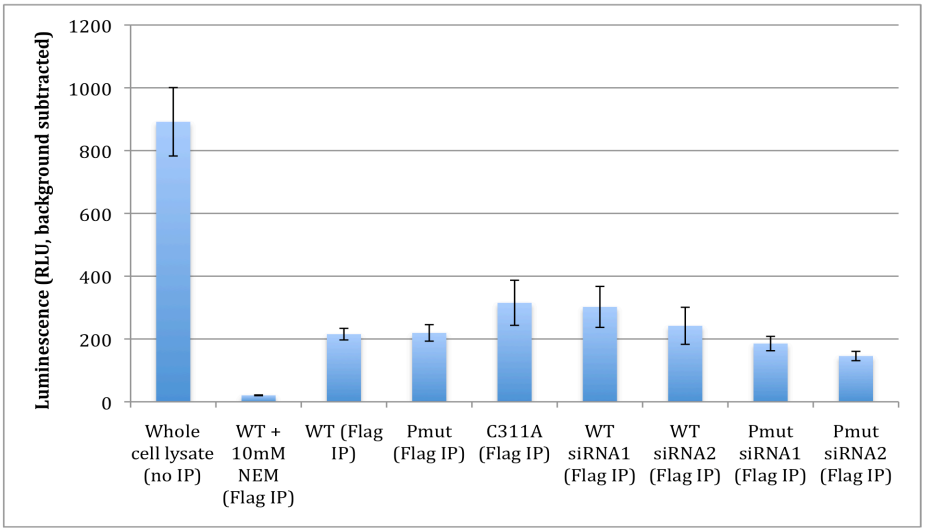

GRK2
80
Usp4
115
Actin
UTC
Nonsilencing control
GRK2 siRNA1
GRK2 siRNA2
GRK2 siRNA1 + WT Usp4
GRK2 siRNA2 + WT Usp4
GRK2 siRNA1 + Pmut Usp4
GRK2 siRNA2 + Pmut Usp4
GRK2 siRNA1 pcDNA3
GRK2 siRNA2 pcDNA3

Figure 16: Usp4 deubiquitinating activity is unaffected by its phosphorylation by GRK2. A) HEK293 cells with or without GRK2 knockdown (as indicated) and overexpression of either WT, P-Mut or C311A Usp4 were flag immunoprecipitated for the deubiquitinating activity assay. One sample of each was run on SDS-PAGE for western blot analysis to demonstrate immunoprecipitation efficiency, in order to determine if fluctuations of activity are due to lack of protein pulled down in the IP or not. We see here that the Ips are approximately even, with slightly less pulled down in samples where GRK2 was knocked down. B) Immunoprecipitates were assayed for deubiquitinating activity using a DUB-Glo assay kit. Whole cell lysate functions as experimental positive control, and adding 10mM NEM to WT Usp4 sample prior to immunoprecipitation serves as a negative control, abolishing the enzyme's activity by deactivating the active cysteine. There is little to no change across all samples, whether overexpressed with WT, P-Mut or C311A and with GRK2 knocked down, indicating that GRK2 does not seem to play a role in Usp4 deubiquitinating activity.

A


IP: Flag
IB: Usp4
115
WT Flag-Usp4
P-Mut Flag-Usp4
C311A Flag-Usp4
WT Flag-Usp4 + siRNA1
WT Flag-Usp4 + siRNA2
P-Mut Flag-Usp4 + siRNA1
P-Mut Flag-Usp4 + siRNA2

B


Luminescence (RLU, background subtracted)
1200
1000
800
600
400
200
0
Whole cell lysate (no IP)
WT + 10mM NEM (Flag IP)
WT (Flag IP)
Pmut (Flag IP)
C311A (Flag IP)
WT siRNA1 (Flag IP)
WT siRNA2 (Flag IP)
Pmut siRNA1 (Flag IP)
Pmut siRNA2 (Flag IP)

of the deubiquitinating activity in the assay are due to actual changes in deubiquitinating activity rather than simply having more or less Usp4 immunoprecipitated.

Figure 16B demonstrates that GRK2 phosphorylation of USP4 has little to no effect on USP4's deubiquitinating activity. Luminescence was recorded as relative light units (RLU), with background subtracted in the figure (for the whole cell lysate positive control, this is the lysis buffer alone, and for the immunoprecipitated samples this is the Flag beads that have been washed 3 times in lysis buffer). The whole cell lysate as a positive control shows that the assay itself is working, as there are many deubiquitinating enzymes present in this sample that will be able to cleave the aminoluciferin-ubiquitin bond to give a luminescent signal. As predicted, adding 10mM NEM to the lysate prior to immunoprecipitation inhibited the deubiquitinating activity of USP4. As we were unsure of how USP4's phosphorylation status affects its deubiquitinating activity, we used a plasmid containing USP4 with site-directed mutations of the two serines (previously made by Jin Yi) and found that mutation of both serines did not alter activity in this assay.

4. DISCUSSION

4.1 Expression of USP4

Obtaining the antibodies specific to USP4 and USP15 and sensitive enough to pick up endogenous expression of the proteins was a key step in determining the role of USP4 in the cell. Because USP4 and USP15 are so similar, it was very important to make sure that the antibodies did not cross-react (Angelats et al., 2003; Baker et al., 1999). A competition assay (incubating the USP4 primary antibody with a recombinant USP4 before incubating the membrane in the primary antibody) may serve as a good experiment to determine which of the possible nonspecific bands are in fact nonspecific. With the prior incubation of the antibody with recombinant protein, any USP4-specific band should disappear. Any band that remains would be nonspecific. When comparing USP4 and USP15 expression in both mice (old and young) and cell lines, there are very few similarities. Whereas USP4 is expressed to a moderate extent in most tissues, USP15 has very low levels of expression, and is expressed in few tissues, as well as few cell lines. The USP4 specific antibody shows a very pronounced band around 70kDa in most of the mouse tissues (FVB/N and C57 alike). Interestingly, the prominence of this band is significantly reduced in the brain, where we also see several more isoforms around the predicted size of USP4 at 108kDa as compared to all of the other tissues (figures 6 to 8). However, these multiple isoforms of similar size are not evident when looking at USP4 protein expression in the cell lines (comparing figures 6 to 9). Cell line U87MG, a human glioblastoma multiforme (GBM) line (Clark et al., 2010) does have low levels of USP4 expression, however we see only one band at the predicted size of 108 kDa (Figure 9). This may in part be due to the fact that the brains examined from the mouse tissues were normal, healthy brains, and not gliomas. However even taking this into consideration, Ertel, et al., have

found significant differences when comparing corresponding cell lines, normal and tumour tissue (Ertel et al., 2006). So, although cell lines provide for a convenient method of experimental investigation, it is more accurate to look at the expression of USP4, and other proteins of interest, within cells that have come directly from the organism, rather than a cell line derived from the tissue.

Similarily, when comparing the lung tissue samples to the lung cancer cell lines, we see a discrepancy. It has been previously found that USP4 is upregulated in small cell lung tumours and acenocarcinomas of the lung, (Gray et al., 1995; Laurell et al., 2009) but looking at USP4 protein expression in A549 cells, an adenocarcinoma cell line, we see little to no USP4 expressed. We see little expression of USP4 in H1299 cells, a non-small cell lung cancer (NSCLC) line, as well. In our mouse tissue samples we see USP4 expression levels to be moderate as compared to the other tissues analyzed. This is the reverse of what is expected, but again may be due largely to the fact that cell lines do not always provide for as accurate a portrayal of protein expression as the actual tumour tissue.

Interestingly, when we look at the PC12 cell line, originating from pheochromocytoma of a rat adrenal medulla, we see that there is a very pronounced band picked up by the USP4 antibody at approximately 80kDa. This may be in agreement with the findings that USP4 is upregulated in adrenocortical tumours. Although not the same tumour types, this may show that tumours originating in the adrenal gland may be influenced by, or may influence the level of USP4 expression. Whether this elevation is cause or effect is yet to be determined. The 80kDa size of the protein is unexpected, but it may be that the protein is being modified in some way in these tumours. On the other hand, it may be nonspecific binding of the antibody to some other protein. In order to determine which is true, knocking down USP4 in these cells and

visualizing whether or not this 80kDa band disappears would be a relatively easy way to find out. If the band disappears, then it is likely that the antibody is in fact picking up a different isoform of USP4 in these PC12 cells.

When looking at USP4 expression in the testes, two very interesting things are visible. Firstly, in the young (3 month old mice, both FVB/N and C57), we see two very strong bands around the predicted size of USP4 (108kDa). These bands almost disappear by the time the mouse has reached age two years (comparing figures 6, 7, and 8). The band at slightly higher molecular weight is gone in the 2 year old mouse, and the lower molecular weight isoform is significantly reduced. This is an interesting find, as there seem to be no other age-related differences when looking at USP4 protein expression, and may indicate a role for USP4 in male reproductive function. Secondly, the fact that we see two different isoforms being expressing is surprising, considering that when looking at USP4 expression at the RNA level, there is only one isoform visible (Gupta et al., 1994) though as we see with the protein expression there is a very high abundance of the transcript. The fact that there is only one mRNA transcript but multiple isoforms of the protein suggests that these isoforms are being processed post-translationally, and are not likely to be splice variants. Although we were surprised to see these two different bands in the testis, it is well documented that many proteins have multiple isoforms in the testes, dependant on post-translational modification (Gorleku and Chamberlain, 2010; Guo et al., 2010; Williams et al., 2007). These modifications may be phosphorylation, ubiquitination, or SUMOylation; all of which have been postulated to be modifying USP4 post-translationally, and could be verified using mass spectrometry.

4.2 Function of USP4

It has been previously shown that knockdown of USP4 affects cell cycle control by interfering with splicing and cell division (Song et al., 2010). This group found that USP4 forms a complex with Sart3 and associates with the spliceosome, regulating it. Conversely, when we knocked down USP4 and looked at its effect on cell cycle, we found that there was no characteristic G2 peak, suggesting a problem during the synthesis (S) phase of the cell cycle (Figure 11). However, as figure 11 shows, there does seem to be cells present at double the fluorescence content of the G1 peak (X-axis – FL2 Channel), indicating that there are diploid cells in the sample. What we see is an accumulation of cells in the G1 phase, with the disappearance of the G2 peak possibly an issue with the date acquisition software able to detect a small enough peak for G2.

USP4 has also recently been implicated in the canonical Wnt signaling pathway (Zhao et al., 2009), prompting us to delve a little deeper into the role it may be playing. Whereas Zhao et al. found that depletion of USP4 activates Beta-catenin-dependent transcription, we demonstrate here that when USP4 is knocked down with our shRNA, Beta-catenin levels drop when compared to untransfected HEK293 cells and HEK293 cells overexpressing wildtype and mutant forms of USP4 (Figure 12). Although we may have initially expected that the catalytic mutant, and possibly the phosphorylation mutant (if phosphorylation were to activate USP4) would have the same effect as knocking down USP4 in the cell, we see that this is not the case. In this case, the overexpressed USP4 is not acting as a dominant mutant in the cell, thus the endogenous USP4 that is present (because USP4 was not knocked down before this overexpression) is expressed at high enough levels to do its' 'job' without being compromised by the overexpressed protein; as is expected, because otherwise Beta-catenin would be

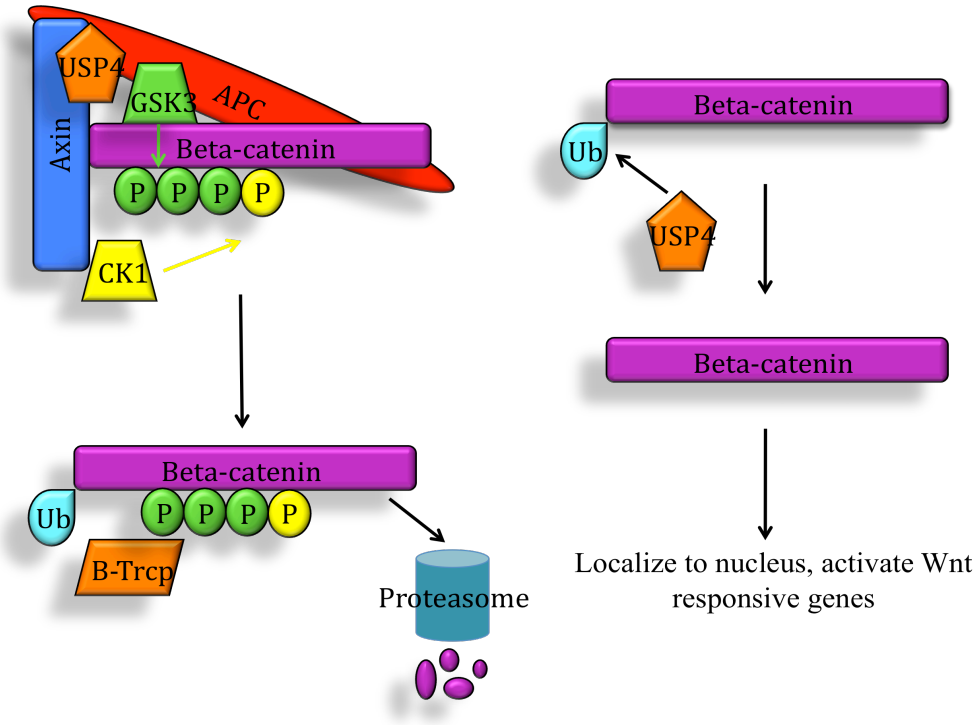
degraded with or without endogenous USP4. One question we may ask, and quite readily answer, is whether or not Beta-catenin levels drop because it is being degraded more quickly (perhaps USP4 removes ubiquitin from Beta-catenin) or is it not being expressed as highly? By knocking down USP4 in cells and performing RT-PCR to determine whether or not the mRNA levels of Beta-catenin are reduced with this knock down, we can answer this question.

Because Beta-catenin levels seem to be affected by USP4, we attempted to co-immunoprecipitate the proteins in order to determine if they interact. Figure 13 shows us that they do not co-immunoprecipitate. The reason may be one of several possibilities: USP4 and Beta-catenin may in fact not interact at all, and any effect of USP4 on B-catenin stability would be indirect. USP4 and Beta-catenin may interact very briefly, as a 'hit-and-run' type of reaction, so to pull down at the right moment enough of the interacting proteins may be improbable. Finally, there may be one or several intermediary proteins linking the two together, so that they may not pull down together, but USP4 may be in close enough proximity to affect Beta-catenin. For example USP4 may be part of the destruction complex, associating with Axin and APC. Were this the case when there is no Wnt ligand to bind to the Frizzled receptor and it's co-receptor, LRP6, to activate the Wnt pathway, this canonical Wnt signaling is turned off, and Beta-catenin would be degraded. USP4 activity may be inhibited in the absence of Wnt ligands by being associated with the destruction complex, and upon activation of canonical Wnt signaling, USP4 may dissociate from the complex, and be free to deubiquitinate any remaining Beta-catenin, precluding its degradation (as depicted in Figure 17). A way to test this would be to determine whether or not USP4 can co-immunoprecipitate with the proteins in the destruction complex (such as Axin, APC, GSK3α/β or CK1) both in the presence of Wnt stimulation, and when there is inhibition of the Wnt signaling pathway using recombinant

Figure 17: Potential model of USP4's involvement in canonican Wnt signaling. With Wnt signaling turned off, USP4 may form part of the destruction complex, associating with Axin and APC and allowing Beta-catenin to be degraded. USP4 activity may be inhibited in the absence of Wnt ligands by being associated with the destruction complex, and upon activation of canonical Wnt signaling, USP4 may dissociate from the complex, and be free to deubiquitinate any remaining Beta-catenin, precluding its degradation.

Wnt signling turned off:

Wnt signaling turned on:



Mesodermal development (Mesd) protein or Sclerostin (SOST) protein, two known inhibitors of the LRP5/6 co-receptor (Lu et al., 2010; Semenov et al., 2005).

4.3 Regulation of USP4

Unpublished data from the Gray lab shows that USP4 is phosphorylated at serines 675 and 680. There have not yet been any studies on USP4 indicating which kinases phosphorylate it, and given that it's main function is still unknown (groups have implicated USP4 in a number of pathways, such as cell division (Song et al., 2010), Wnt signaling (Zhao et al., 2009), and associating with a number of proteins, including proteins within the spliceosome (Nottrott et al., 2002), Ro52 (Di Donato et al., 2001), and $A_{2A}R$ (Milojevic et al., 2006; Toews, 2006)), we have yet to determine the effect of this phosphorylation on the protein itself.

To determine which kinase is phosphorylating USP4 at the predicted sites, we sent a GST-tagged peptide to the laboratory of Dr Rob Screaton for a kinase screen, which is an *in vitro* assay using recombinant kinases to determine whether or not they will phosphorylate USP4. The primary screen generated 24 positive hits (table 2). Additional peptide was submitted for a secondary screen: a focused screen looking at only the positive hits from the primary results to look for any whose activity was reproducible. The secondary screen brought back three positive hits, that were graded at high, medium and low for their USP4:MBP ratio, which indicates specificity of the phosphorylation (table 3). A high ratio shows high specificity, and a low ratio shows low specificity. Of the three hits, MARK4 and TTBK1 had low USP4:MBP ratios. Interestingly, GRK2 (or ADRBK1) had a high specificity ratio, and like USP4, has also been implicated in canonical Wnt signaling; it is thought to down-regulate the signaling cascade (Wang et al., 2009).

We ran into problems when knocking down GRK2 in HEK293 cells. Both siRNAs knocked down GRK2 very efficiently (Figure 15), however further perturbation of the cell by the overexpression of either a vector containing USP4 or even an empty vector control, significantly decreased the knockdown efficiency. This posed a problem when moving onto the next experiment, of determining the effect of GRK2 phosphorylation on USP4. After attempting to knock down GRK2 in HEK293 cells, we performed a DUB activity assay, which can indicate whether phosphorylation of USP4 increases, decreases, or has no effect on its enzymatic activity. As Figure 16 demonstrates, USP4 deubiquitinating activity seems to be unaffected by its phosphorylation by GRK2. However, because Figure 15 shows that GRK2 knockdown efficiency decreases upon overexpression of USP4, we are unable to make conclusions as to how GRK2 is affecting USP4 in this assay. Surprisingly, the catalytically inactive mutant of USP4 (C311A) does not have reduced activity in the assay as compared to the wildtype and phosphorylation mutant USP4. This is unexpected, as it as been previously shown that this mutant does have decreased activity by enzymatic analysis in bacterial cells (PhD Thesis, Paola Blanchette) and this vector has been sequenced, confirming the mutation. A possible explanation for this may be USP4 is able to associate in a complex with another deubiquitinating enzyme that is able to co-immunoprecipitate with USP4 and cleave the aminoluciferin-ubiquitin bond, generating luminescence. Because this assay is not specific to USP4, but shows general deubiquitination activity, we cannot decipher with this assay whether or not it is USP4 responsible for the activity. Intriguingly, USP15 is also known to function in the Wnt signaling pathway as a component of the COP9 signalosome, but we have no evidence that USP4 and USP15 physically associate (Huang et al., 2009). As Huang et al., have found that the COP9 signalosome, complexed with CRLs (cullin-RING Ub ligases) and the

proteasome, associates with the Beta-catenin destruction complex (Huang et al., 2009), and if our hypothesis is correct in that USP4 associates with the destruction complex, this may indicate that the deubiquitinating activity of the catalytically inactive USP4 mutant is in fact coming from USP15 which is able to be pulled down with USP4 as a part of this supercomplex. In this instance, when we pull down USP4 using FLAG beads, we may also be pulling down USP15 from this huge complex. This could be determined by seeing if USP4 and USP15 will co-immunoprecipitate. Because Huang et al., found that upon stimulation by Wnt, the complexes dissociated, it may also be useful to determine whether or not USP4 and USP15 co-immunoprecipitate after Wnt treatment, as well as performing this deubiquitinating activity assay after the cells have been stimulated by Wnt to confirm the intermingling of these two hypotheses. What role GRK2 plays in this model remains to be elucidated.

Finally, we have recently ordered a USP4 knockout mouse (which will be in the form of a gene-trap mutant) that will be useful in order to resolve some of the outstanding issues, such as the effect of USP4 on specific tissues and cell types in the mouse. Because of the strong expression and multiple isoforms of USP4 in the testis of young mice, which is depleted as the mouse ages, perhaps these mice will have reduced sterility? And possibly because USP4 upregulation has been found in certain cancers, maybe the lack of USP4 will cause increased cancer resistance. This mouse will also be useful in further determining the specificity of the USP4 chicken antibody that we had generated. We will be able to confirm which bands in the western blots are indeed USP4, and which are nonspecific binding. This mutant will also shed light on the effect of USP4 on Beta-catenin stability, and USP4's role in the Wnt signaling pathway.

5. Conclusions

To date, studies have identified many possible interacting partners, substrates, and pathways of which USP4 may be involved. But none have looked at expression of USP4 within the organism, to indicate where and how the organism may be affected by mutations. Similarly, none have looked at how USP4 is regulated, which may lead to a better understanding of its role in health and disease. The present study indicates that there are differences in expression of USP4 among tissues, and that expression changes in the testis of male mice when the organism ages. We have also demonstrated involvement of USP4 in canonical Wnt signaling as has recently been reported, albeit in a different light. Additionally, we have identified a kinase that phosphorylates USP4 in vitro, which is a novel finding. With the present results, we have been able to incorporate USP4 into a model, potentially clarifying the function of USP4 in canonical Wnt signaling, and how its function is regulated. Further studies are necessary to confirm the new hypotheses generated by this work.

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7. CONTRIBUTIONS OF COLLABORATORS

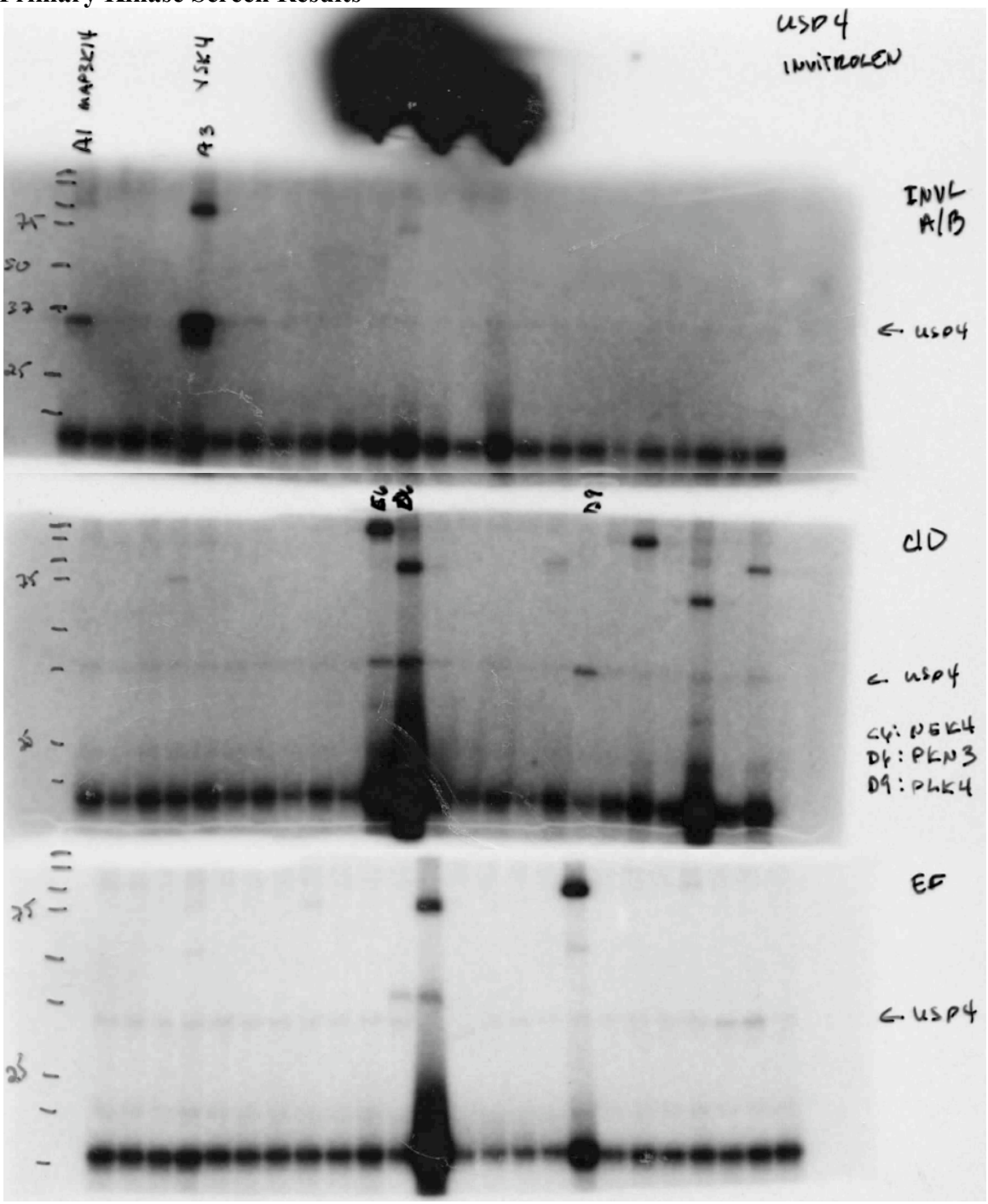
Collaborated with Dr. Robert Screaton for the kinase screens. I generated the GST-USP4 peptide and brought it to Dr. Screaton's lab, which performed the kinase screen and provided me with the results.

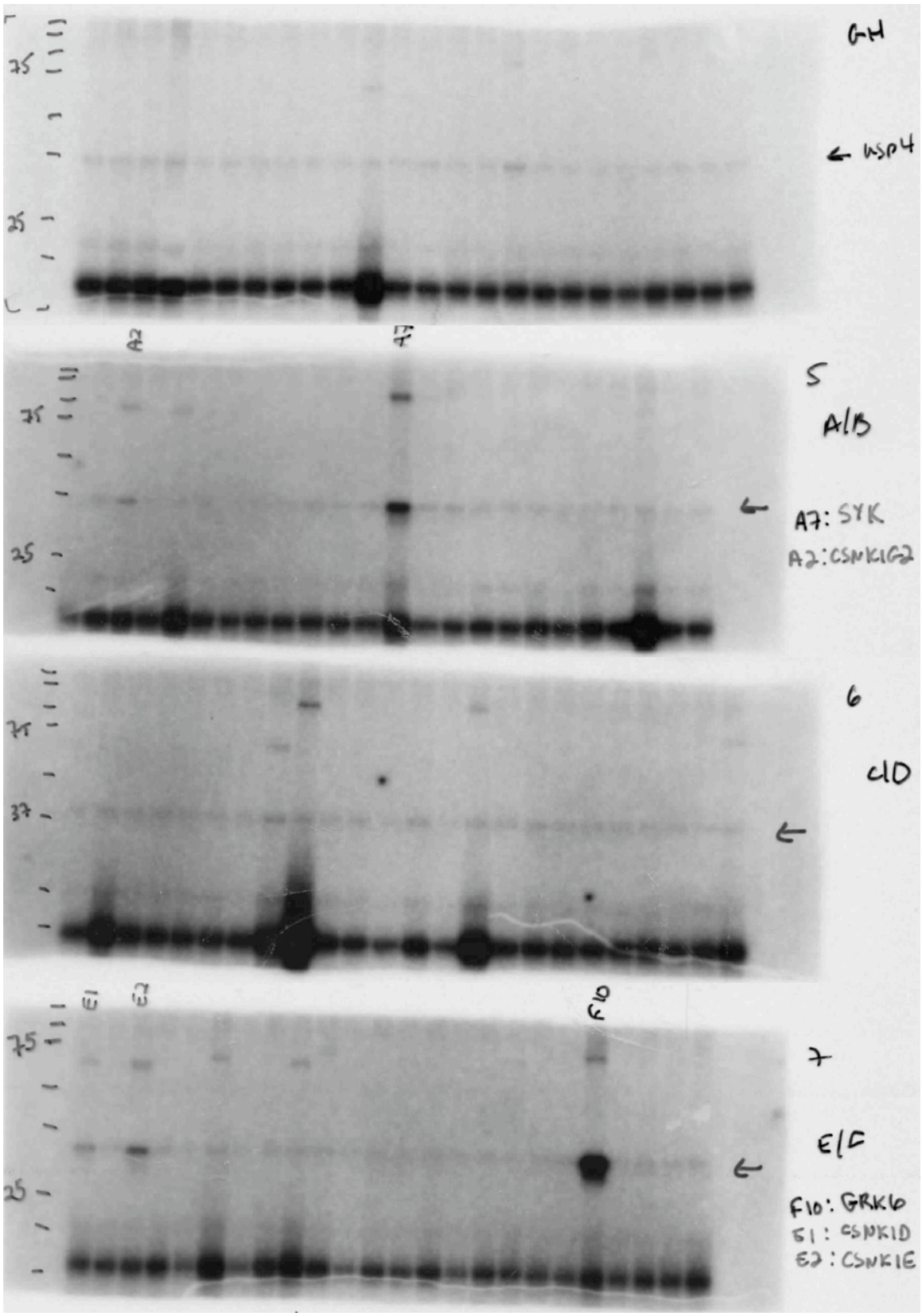
Previous students in the lab created some of the constructs used in my thesis. The WT-FLAG USP4 was made by Erin Twomey, and the phosphorylation (PMut) FLAG-USP4 was made by Jin Yi.

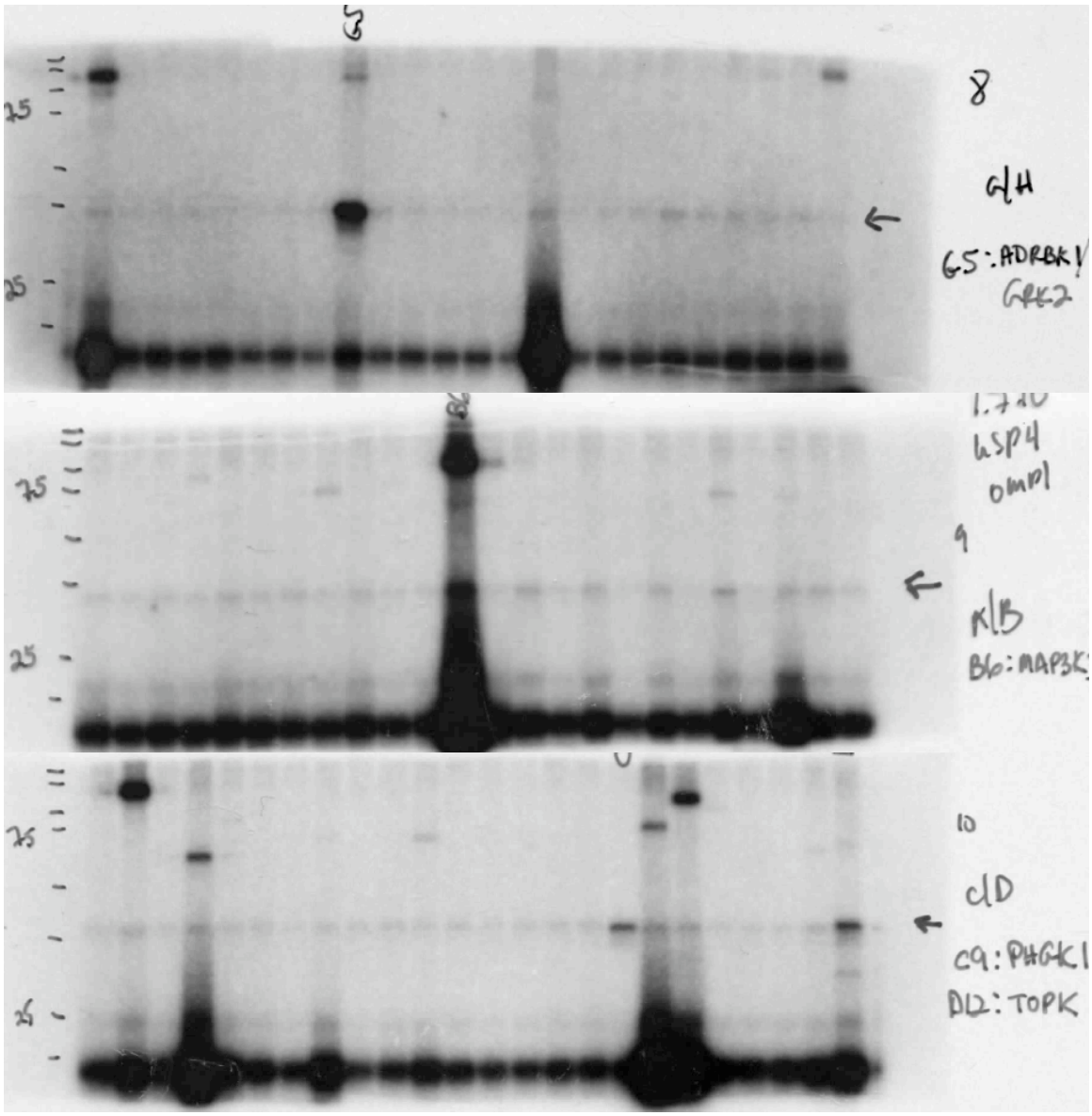
Dr. Bruce McKay helped me with analyzing the flow data, and Jeff Hamill of the McKay lab helped me obtain the flow results.

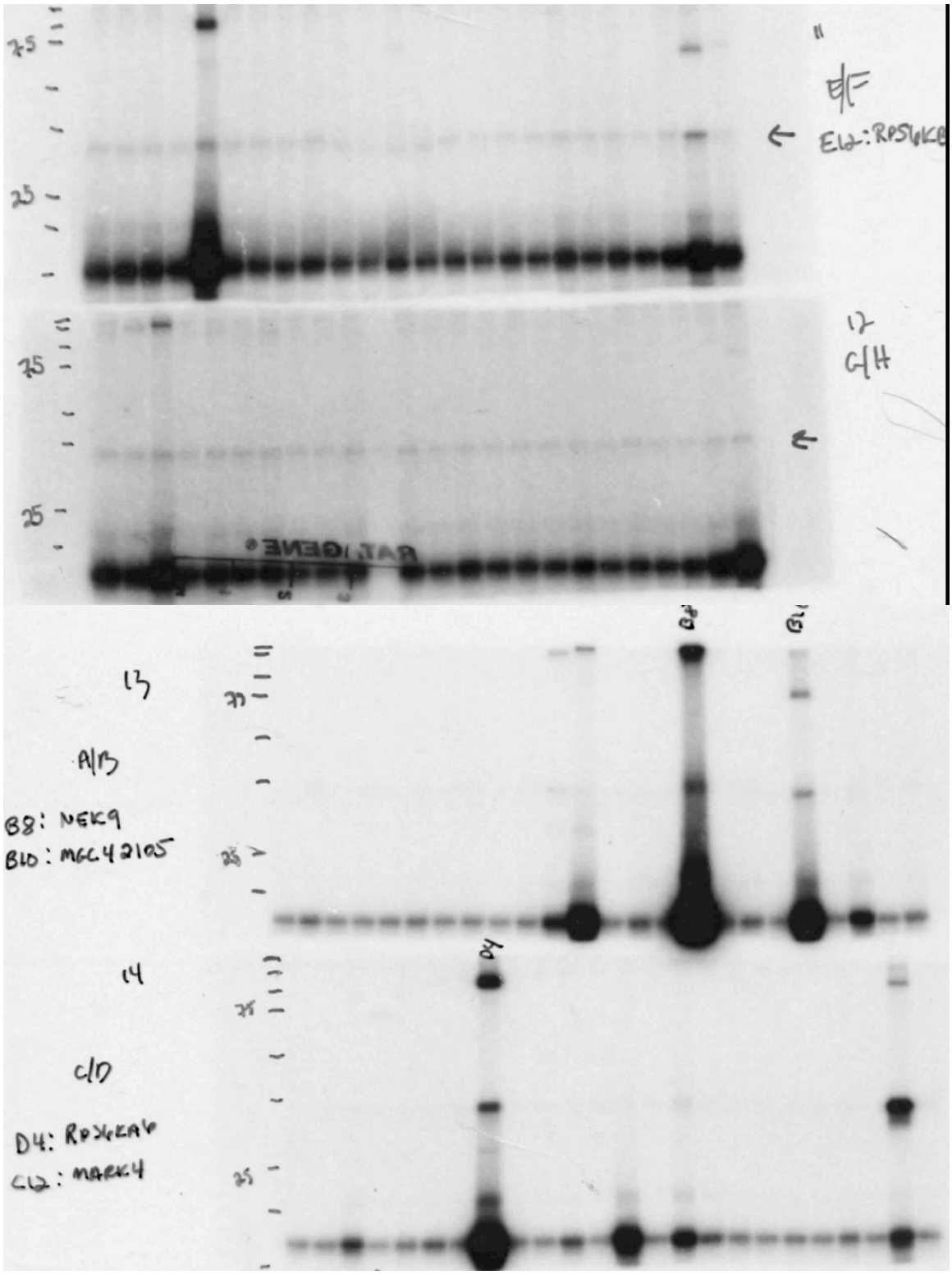
8. Appendix

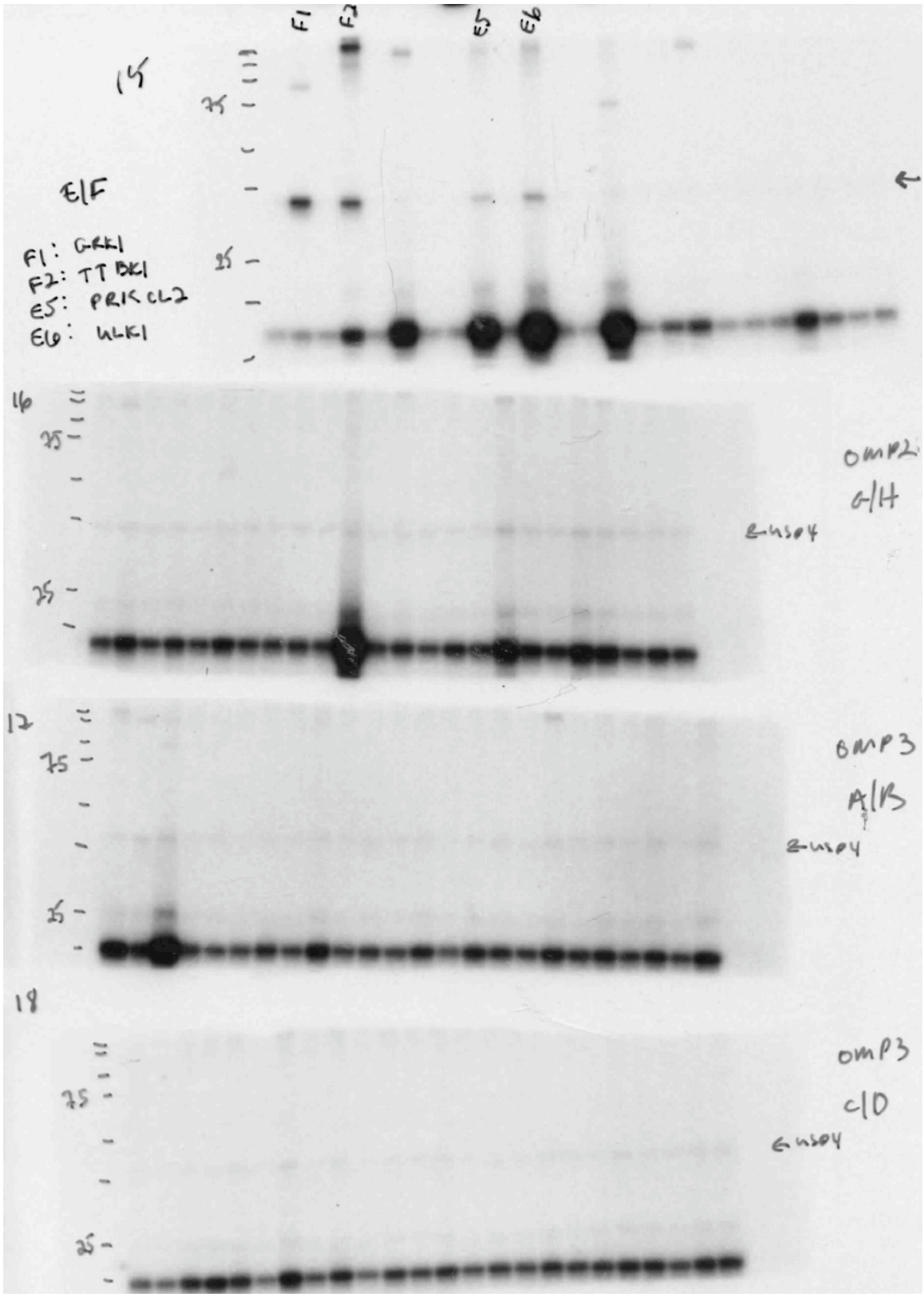
Primary Kinase Screen Results

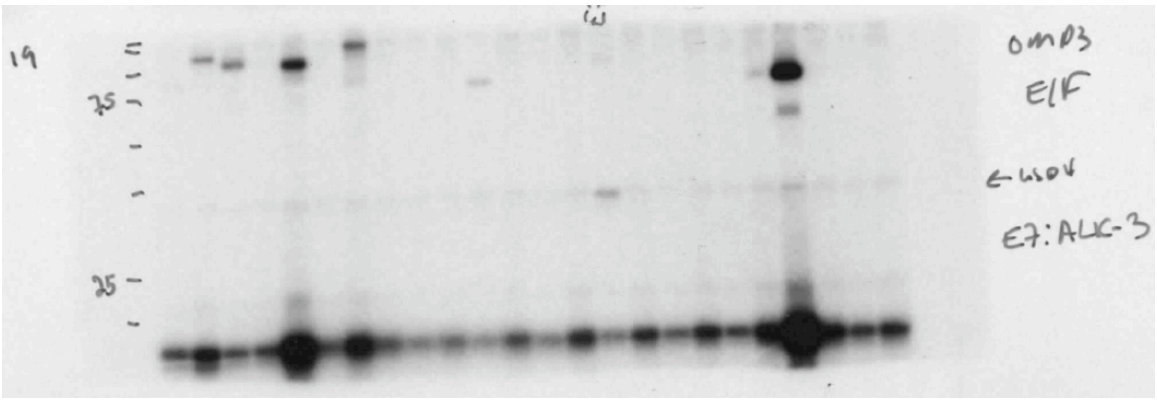



GH
WSP4
75
25
A2
A7
5
A/B
A7: SYK
A2: CSNK1G2
75
25
6
C1D
75
37
E1
E2
F10
7
E/F
75
25
F10: GRK6
E1: CSNK1D
E2: CSNK1E


8
G|H
G5: ADRBK1/
GRK2
9
K|B
B6: MAP3K
10
C|D
C9: PHGK1
D12: TOPK

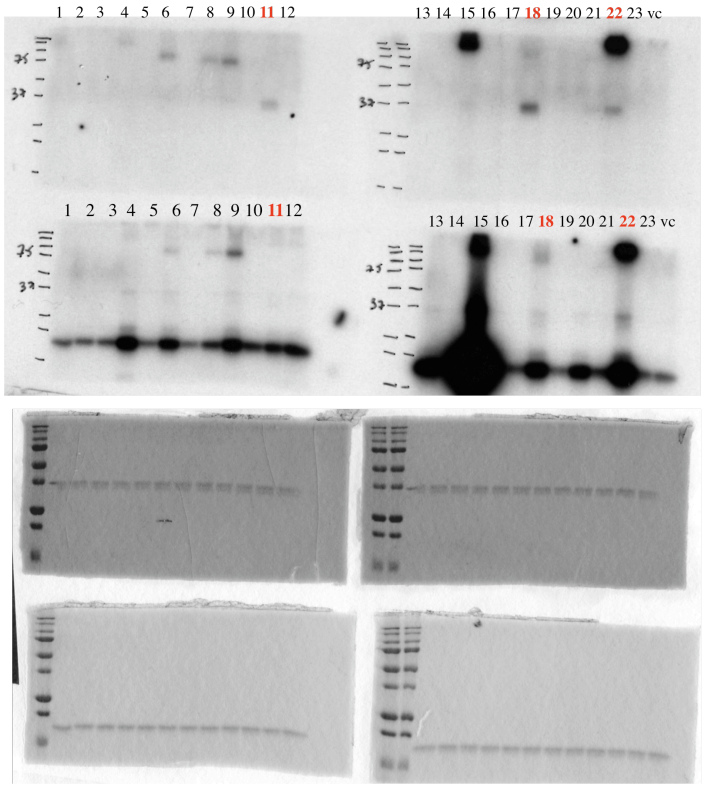

75
25
E12: RPS6KB
12
G1H
75
25
13
A1B
B8: NEK9
B10: MGC42105
14
C1D
D4: RPS6KA6
C12: MARK4


15
F1
F2
E5
E6
75
25
E1F
F1: GRK1
F2: TTBK1
E5: PRKCL2
E6: WNK1
16
75
25
OMP2
G/H
anti-V5
12
75
25
OMP3
A/B
anti-V5
18
75
25
OMP3
C/D
anti-V5



Secondary Kinase Screen Results

1 MAP3K14
2 YSK4
3 NEK4
4 PKN3
5 PLK4
6 CSNK1G2
7 SYK
8 CSNK1D
9 CSNK1E
10 GRK6
11 ADRBK1
12 MAP3K3
13 PHGK1
14 TOPK
15 NEK9
16 MGC42105
17 RPS6KA6
18 MARK4
19 PRKCL2
20 ULK1
21 GRK1
22 TTBK1
23 ALK-3
24 vec.cont



9. Curriculum Vitae – SOPHIE BASTARACHE

ACADEMIC HISTORY

2008-2011: **University of Ottawa**
Department of Biochemistry, Microbiology and Immunology
Program: Biochemistry – Human and Molecular Genetics
451 Smyth Road, Ottawa
K1H 8M5
Master of Science
Graduation Date: August 2011

2003-2008: **University of Ottawa**
Department of Biochemistry, Microbiology and Immunology
Program: Biochemistry with Coop
451 Smyth Road, Ottawa
K1H 8M5 Canada
Bachelor of Science
Graduation Date: June 2008

SCHOLARSHIPS

University of Ottawa Entrance Scholarship ($1500)
University of Ottawa Graduate Student Admission Scholarship ($12500)

PROFESSIONAL EXPERIENCE

2008-2011: **The Ottawa Hospital Cancer Centre,** Ottawa, Ontario
University of Ottawa
Masters Student
Supervisor: Dr. Douglas Gray
Project: Characterization of Usp4 expression and activity in mammalian tissues

2007-2008: **Apoptosis Research Centre,** Ottawa, Ontario
Children's Hospital of Eastern Ontario
Coop/Honours Student
Supervisor: Dr. David Stojdl
Project: Comparing evasion of the antiviral response by oncolytic viruses in cancer cell lines

2006: **Apoptosis Research Centre,** Ottawa, Ontario
Children's Hospital of Eastern Ontario
Coop Student
Supervisor: Dr. Peter Liston
Project: Creating Beclin-1 constructs to study autophagy in cancer cells and Beclin 1 promoter studies

2006: **Boehringer Ingelheim (Canada) Ltée,** Laval, Québec
Research & Development
Coop Student
Supervisor: Colette Bouchard
Project: HPLC method development for separation of various HIV and Hepatitis C projects

RESEARCH SKILLS

Experience in Various Molecular and Biochemical Techniques including:
Tissue culture of mammalian tumor, primary human cell lines, Mammalian cell transformation, transduction and transfection, Immunofluorescence, Immunoprecipitation, Western blot analysis, DNA and RNA Isolation, Cloning, Sub-cloning, Gel Electrophoresis, PCR, Interferon Assay, Plaque Assay

ORAL PRESENTATIONS

1. Coop project presentation at Boehringer-Ingelheim (Canada) Ltée. HPLC Method Development for separation of various HIV and HCV projects (2006)
2. WIP presentation at the Apoptosis Research Center. Creating Beclin-1 constructs to study autophagy in cancer cells (2006)
3. BCH 4932 Seminar presentation. How the Rabies P Protein Interacts with STAT1 to Inhibit the Interferon Pathway (2007)
4. BCH 4932 Seminar presentation. Comparing Viral Evasion of the Host Cell's Antiviral Response (2008)
5. BCH 8105 Seminar presentation. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy (2009)
6. BCH 8104 Seminar presentation. Progenitors of skeletal muscle satellite cells express the muscle determination gene, MyoD (2009)
7. OHRI Research day and BMI poster day presentations. Characterization of Usp4 expression in mammalian tissues (2009)
8. Graduate Student Seminar. Characterization of Usp4 and Usp15 expression in mammalian tissues (2010)