# IDENTIFICATION OF NOVEL SUBSTRATES AND BINDING PARTNERS OF SPECKLE- TYPE POZ PROTEIN ADAPTER FROM CULLIN3- RING E3 UBIQUITIN LIGASE COMPLEX

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**ABSTRACT**

Speckle type POZ protein (SPOP) is a ubiquitin E3 ligase protein adapter found to be highly associated with prostate cancer. SPOP prostate cancer mutations were identified to be located within the Meprin and TRAF- C homology (MATH) domain of the protein, which is responsible for substrate recognition and interaction. This study focuses on identifying novel substrates/ binding partners of SPOP and characterizing their involvement in cancer initiation and progression. Due to the high sequence and structural similarity to the MATH domain of Ubiquitin- Specific Protease 7 (USP7) it is possible that there are many overlapping substrates/interacting partners between these two proteins. Eleven novel cellular and viral proteins that interact with SPOP were identified. The cellular proteins were shown to be destabilized upon SPOP over expression. Prostate cancer SPOP mutants showed loss of interaction with their substrates. These novel SPOP substrates/ binding partners bring insight into its involvement in cellular pathways and cancer development.

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## LIST OF ABBREVIATIONS

AA Amino acid

| | | |
|---|---|---|
| A | Ala | Alanine |
| C | Cys | Cysteine |
| D | Asp | Aspartic acid |
| E | Glu | Glutamic acid |
| F | Phe | Phenylalanine |
| G | Gly | Glycine |
| H | His | Histidine |
| I | Ile | Isoleucine |
| K | Lys | Lysine |
| L | Leu | Leucine |
| M | Met | Methionine |
| N | Asn | Asparagine |
| P | Pro | Proline |
| Q | Gln | Glutamine |
| R | Arg | Arginine |
| S | Ser | Serine |
| T | Thr | Threonine |
| V | Val | Valine |
| W | Trp | Tryptophan |
| Y | Tyr | Tyrosine |

| | |
|---|---|
| 53BP1 | p53 binding protein 1 |
| AKT | Protein kinase B |
| ATM | Ataxia telangiectasia mutated |
| ATR | Ataxia telangiectasia and Rad3-related protein |
| ATP | Adenosine triphosphate |
| AR | Androgen receptor |
| BTB | Broad- complex, Tramtrack and Bric a Brac |
| BRCA1 | Truncated breast and ovarian cancer susceptibility protein 1 |
| CHFR | Checkpoint with forkhead and ring finger domains |
| CHK1 | Checkpoint kinase 1 |
| CRL | Cullin- Ring ligase |
| CRL3 | Cullin 3 RING- box based ubiquitin E3 ligase |
| Cul1 | Cullin 1 |
| Cul3 | Cullin 3 |
| CXCL2 | C-X-C Motif Chemokine Ligand 2 |
| DAXX | Death domain associated protein |
| DDIT3 | DNA Damage-Inducible Transcript 3 |
| DNA | deoxyribonucleic acid |
| DDR | DNA damage response |
| E1 | Ubiquitin E1 activating enzyme |
| E2 | Ubiquitin E2 conjugating enzyme |
| E3 | Ubiquitin E3 ligase |
| EBNA1 | Epstein-Barr nuclear antigen 1 |
| EBNA2 | Epstein-Barr nuclear antigen 2 |
| EBV | Epstein-Barr virus |
| EGFR | Epidermal growth factor receptor |
| ERG | ETS Transcription Factor ERG |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase |
| GST | Glutathione S- transferase |
| H2A | Histone protein 2A |
| H2B | Histone protein 2B |

| | |
|---|---|
| HCMV | Human cytomegalovirus |
| HDM2 | Human double minute 2 |
| HECT | Homologous to the E6-AP carboxyl terminus |
| HSV | Herpes simplex virus |
| HPV | Human Papillomavirus |
| HR | Homologous recombination |
| HRP | Horseradish peroxidase |
| IFN | Interferon |
| inF2 | Inverted Formin, FH2 And WH2 Domain Containing |
| IPTG | Isopropyl β-D-1-Thiogalactopyranoside |
| IRF | Interferon Regulatory Factor |
| MATH | Meprin and TRAF homology |
| MDM2 | Murine double minute 2 |
| MDMX | Murine double minute X |
| NHEJ | non- homologous end joining |
| NKX3.1 | NK3 Homeobox 1 |
| NMR | Nuclear Magnetic Resonance |
| PCR | Polymerase chain reaction |
| PcG | Polycomb group |
| PLZF | Promyelocytic leukemia zinc finger |
| PRC1 | Polycomb repressive complex 1 |
| PTEN | Phosphatase and Tensin Homolog |
| PTM | Post- translational modification |
| Pp71 | protein phosphatase 71 |
| PAGE | polyacrylamide gel electrophoresis |
| RAD51 | RAD51 recombinase |
| RING | Really interesting new gene |
| SEDT2 | SET Domain Containing 2 |
| SENP7 | SUMO Specific peptidase 7 |
| RNF6 | Ring finger protein 6 |
| SCF | Skp/ Cullin/ F-Box |

| | |
|---|---|
| SDS | Sodium Dodecyl Sulfate |
| SUMO | Small Ubiquitin-related Modifier |
| SPOP | Speckle type POZ protein |
| TB | Terrific broth |
| TFPT | TFC3 fusion partner |
| TRAF | Tumor necrosis factor receptor-associated factor |
| Ub | Ubiquitin |
| UBA1 | Ubiquitin- like modifier- activating enzyme 1 |
| UBC | Ubiquitin- conjugating |
| UBD | Ubiquitin binding domain |
| UBE1L2 | Ubiquitin- like modifier- activating enzyme 6 |
| UbE2E1 | Ubiquitin conjugating enzyme E2E 1 |
| UbE2E2 | Ubiquitin conjugating enzyme E2E2 |
| UbE2E3 | Ubiquitin conjugating enzyme E2E3 |
| Ubl | Ubiquitin-Like |
| USP7 | Ubiquitin specific protease 7 |
| VIRF1 | Viral Interferon Regulatory Factor 1 |
| VIRF2 | Viral Interferon Regulatory Factor 2 |
| VIRF3 | Viral Interferon Regulatory Factor 3 |
| VIRF4 | Viral Interferon Regulatory Factor 4 |
| VEGFR2 | Vascular endothelial cell growth factor receptor 2 |

## 1. INTRODUCTION

Post - translational modifications (PTMs) are enzymatic alterations to proteins which result in increased functional diversity. PTMs regulate protein activities through altering protein subcellular localization, modifying protein-protein interactions and determining the fate of proteins (Filtz et. al, 2014). PTMs such as phosphorylation, methylation, acetylation, ubiquitination or sumoylation, play critical roles in gene regulation, cell signaling, growth and development (Prabakaran et. al, 2012). Recent research demonstrates that PTMs, such as ubiquitination, are essential in regulating many key factors in cancer (Krueger et. al, 2006). The initiation and progression of cancer is highly associated with dysregulation of tumor suppressors and proto-oncogenes. Multiple mutations in genes encoding for various enzymes of the ubiquitination pathways are found to cause or contribute to tumorigenesis and tumor progression (Gallo et. al, 2017). This suggests that understanding the tumorigenic mechanisms caused by mutations in components of the ubiquitination pathway could allow a better understanding of cancer initiation and development, and ultimately contribute to developing cancer treatments.

### 1.1 Ubiquitination

Ubiquitination is a post- translational modification best known for its function in targeting proteins for degradation. It also plays an essential role in regulating a variety of cellular processes including cell proliferation, differentiation, signal transduction and transcriptional regulation (Hershko et. al, 1998). Ubiquitination occurs when the C-terminal glycine 76 of ubiquitin (Ub) is covalently attached to a lysine residue on the substrate proteins. Ub is a small protein consisting of 76 amino acids and is 8.5kDa in

size (Haglund et. al, 2005). Found in most eukaryotic organisms, Ub is highly structurally and functionally conserved. When first discovered in live cells, Ub was found to be expressed ubiquitously in all cell types including yeast and bacteria (Goldstein et. al, 1975). It was discovered in T and B cells in the immune system and given the name UBIP, ubiquitous immunopoietic polypeptide, because it was believed to have immunopoietic functions. However, it was later discovered that UBIP is not present in bacteria cells and did not have immunopoietic functions (Ciechanover et. al, 2005). The true function of Ub was revealed upon the discovery of ATP- dependent proteolytic activities which the addition of an 8.5 kDa protein in the substrate protein was found to be essential for its proteasomal degradation. In the presence of ATP, Ub covalently attaches to H2A and H2B histone proteins. Although H2A and H2B are not degraded, this alteration is consistent with the previously identified proteolytic pathway (Ciechanover et.al, 2005). It was later established as the ubiquitin- proteasome system, which starts with the conjugation of Ub, and ends with ATP dependent protein degradation (Wilkinson, 2005).

As mentioned previously, Ub demonstrates high levels of sequence and structural evolutionary conservation. Its high sequence similarity was previously demonstrated in yeast and plants, in which a vector expressing *Arabidopsis thaliana* Ub is fully functional when introduced to *Saccharomyces cerevisiae* (Ling et. al, 2000). Belonging to the ubiquitin superfamily, there are several features that are structurally conserved for functional purposes **(figure 1.1**). Ub consists of a hydrophobic core, which enables its interaction with the proteasome (Kulathu et. al, 2012) and mediates endocytosis where Ub acts as a crucial sorting signal for protein internalizations (Piper et. al, 2014). The surface region of Ub is important for mediating protein- protein interaction through an

extended network of hydrogen bonds. Overall, the compact structure of Ub aids its function as well as ensures high levels of heat stability and pH resistance (Callis et. al, 2014).

Taking a closer look at the structure of Ub, there is a unique fold called the β- grasp fold, that consists of a β-sheet with 5 anti-parallel beta-strands as well as an alpha helix which connects β-strands 2 and 3 (ββαββ) (Hochstrasser et. al, 2009; Burroughs et. al, 2012). The β- grasp fold is also present in other small proteins named ubiquitin- like proteins (Ubls), which are also involved in various cellular processes including cell cycle progression and immune response (Welchman et. al, 2005). Another important feature of the Ub structure is the two glycine residues at the C- terminal tail. These two residues are required for Ub's conjugation to its target proteins by forming an isopeptide bond between the glycine residues of Ub and the lysine residues of the target protein (Vijay- Kumar et. al, 1987). Another important feature of Ub includes the seven lysine residues, K6, K11, K27, K29, K33, K48 and K63, which are essential for the formation of polyubiquitin chains connecting to the ubiquitin lysine residues bound to target proteins. In addition, Ub interacting proteins also demonstrate high levels of evolutionary conservation. Many proteins consist of a distinct ubiquitin binding domain (UBDs), which serve to contact the surface of Ub (Husnjak et. al, 2012).

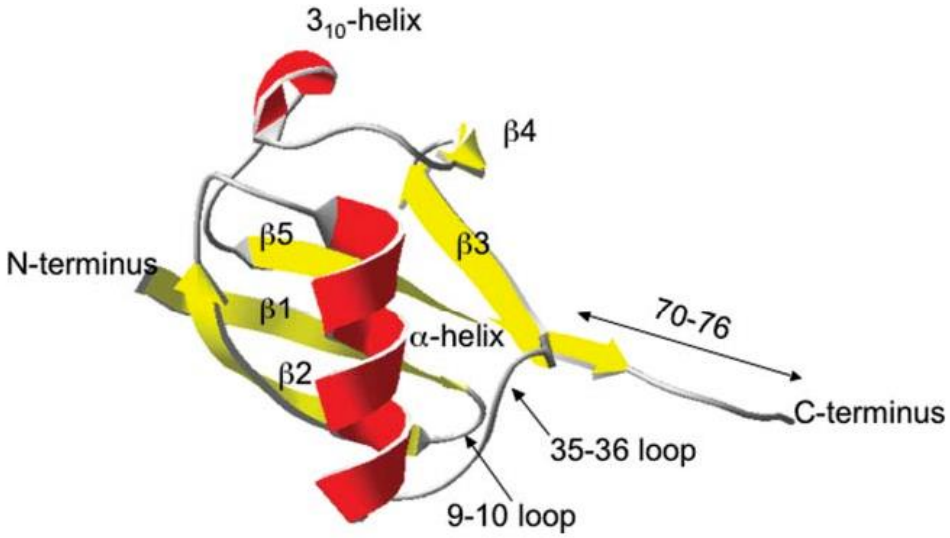



Figure 1.1 Ubiquitin protein structure, adapted from Jackson et.al, 2006. Ubiquitin is a small 76- residue protein. The crystal structure (PDB ID: 1UBQ) above shows the secondary along with the tertiary structure, which includes five β-strands (yellow) as well as an alpha- helix between the second and third β-strands.

Ub is attached to target proteins through an E1- E2- E3 enzymatic reaction cascade which includes activation, conjugation and ligation (**figure 1.2**). This catalytic event begins with an E1 activating enzyme, which uses ATP to form a high energy thioester bond, catalyzing the acyl-adenylation of the Ub at its carboxy- terminal carboxylate (Ciechanover et. al, 2005; Jin et. al, 2007). The Ub moiety is now attached to the cysteine residue of E1. Prior to the transfer to E2, a second Ub molecule is non-covalently bound to E1 which causes a conformational change of E1 which favors the transfer of the Ub to E2 (Schulman et. al, 2009). To date, there are only two E1s identified, ubiquitin- like modifier- activating enzyme 1 (UBA1) and Ubiquitin- like modifier- activating enzyme 6 (UBE1L2). They are responsible for ubiquitin activation for all pathways. In

comparison, there are around 40 E2s known. These E2 Ub- conjugating enzymes then interact with both E1 and the Ub in order to transfer the attached Ub molecules to E2 active site on a cysteine residue (Stewart et. al, 2016). All E2s contain a similar core, Ub conjugating catalytic domain (UBC), which catalyze the transfer of ubiquitin.

Lastly, E3 ubiquitin ligase catalyzes the transfer of Ub to its selected substrate proteins (Komander & Rape, 2012). E3 enzyme can bind directly or indirectly to the substrate to facilitate ubiquitin transfer depending on the type of E3s. In the entire human proteome, there are approximately 600 known E3 ligases. They are divided into three classes based on their catalytic domains: RING (Really Interesting New Gene), HECT (Homology to E6AP C- terminus) and RING-like or U- box. In HECT-E3 ligases, the HECT domain catalyzes the binding of Ub to its active site and binds to the substrate. The highly conserved HECT domain consists of 30 residues and is capable of accepting Ub from E2s and forms a thiol- ester bond at the cysteine residue in its active site for Ub transfer (Huibregtse et. al, 1995). In comparison, the RING domain in RING- E3 ligases transfers the ubiquitin to substrate by bringing the E2 complex to close proximity to the substrate and catalyze its ligation. Further reaction mechanism of RING domain E3s will be discussed in later sections.

Another family of RING domain E3 ligase discovered is the RBR (RING between RING) family with 14 members (Marin et. al, 2002). The RBR E3s can catalyze the direct transfer of Ub and recruit E2s. RBRs consist of three domains and two of the domains are first identified to contain multiple cysteine residues, then a third domain was discovered in between the RING sequence which also contains multiple cysteines. These cysteine residues play an important role in coordination of zinc ions, which mediate

ubiquitin transfer (Spratt et. al, 2014). In terms of function, the C- terminal domain is required for catalysis (named Rcat) and the central domain (BRcat) is named benign-catalytic domain which lacks a cysteine residue, so it does not have catalytic functions (Marin et. al, 2004).

Depending on the type of ubiquitin linkage and chain, the substrate is subjected to a different fate as well as function (Weissman et. al, 2001). A complex can be modified with a single Ub moiety, mono-ubiquitination, or poly- ubiquitinated which is when the substrate is modified with two or more Ub. In monoubiquitination, one Ub is attached to one lysine residue of the substrate and is often found to impact transcriptional regulation as well as protein trafficking. One example is the monoubiquitination of histone H2A by RING1B of the Polycomb Repressive Complex (PRC1). Ubiquitination of histone H2A leads to chromatin compaction, which results in gene silencing (Abdouh et al. 2015). If the substrate consists of multiple lysine residues, monoubiquitination can also occur multiple times which often leads to endosome formation and lysosomal degradation (Herrmann et. al, 2007). As for polyubiquitination, Ubs are conjugated as polymeric chains. Ubiquitin polymers can be attached of one or multiple lysine residues (K6, K11, K27, K29, K33, K48, and K63) which results in diverse range of functions. In terms of function, polyubiquitination determines the fate of the protein specifically based on which of the seven lysine residues is polyubiquitinated. For example, a substrate would be subjected to protein degradation through K48 as it is targeted by the proteasome and its proteolytic functions. Substrates polyubiquitinated at K29 are known to subject to lysosomal degradation and K63 polyubiquinated is involved in cellular signaling pathways including DNA damage repair (Chan et. al, 2001).

Majority of the substrates are subjected towards proteasomal degradation. An established route of degradation is through the 26S proteasome, which is a large ATP dependent protease that uses polyubiquitin chains as a marker (Varshavsky et. al, 1997). Overall, ubiquitination has important functions in cell cycle progression, histone modification, transcriptional regulation, and many other cellular processes (Nath et. al, 2009).

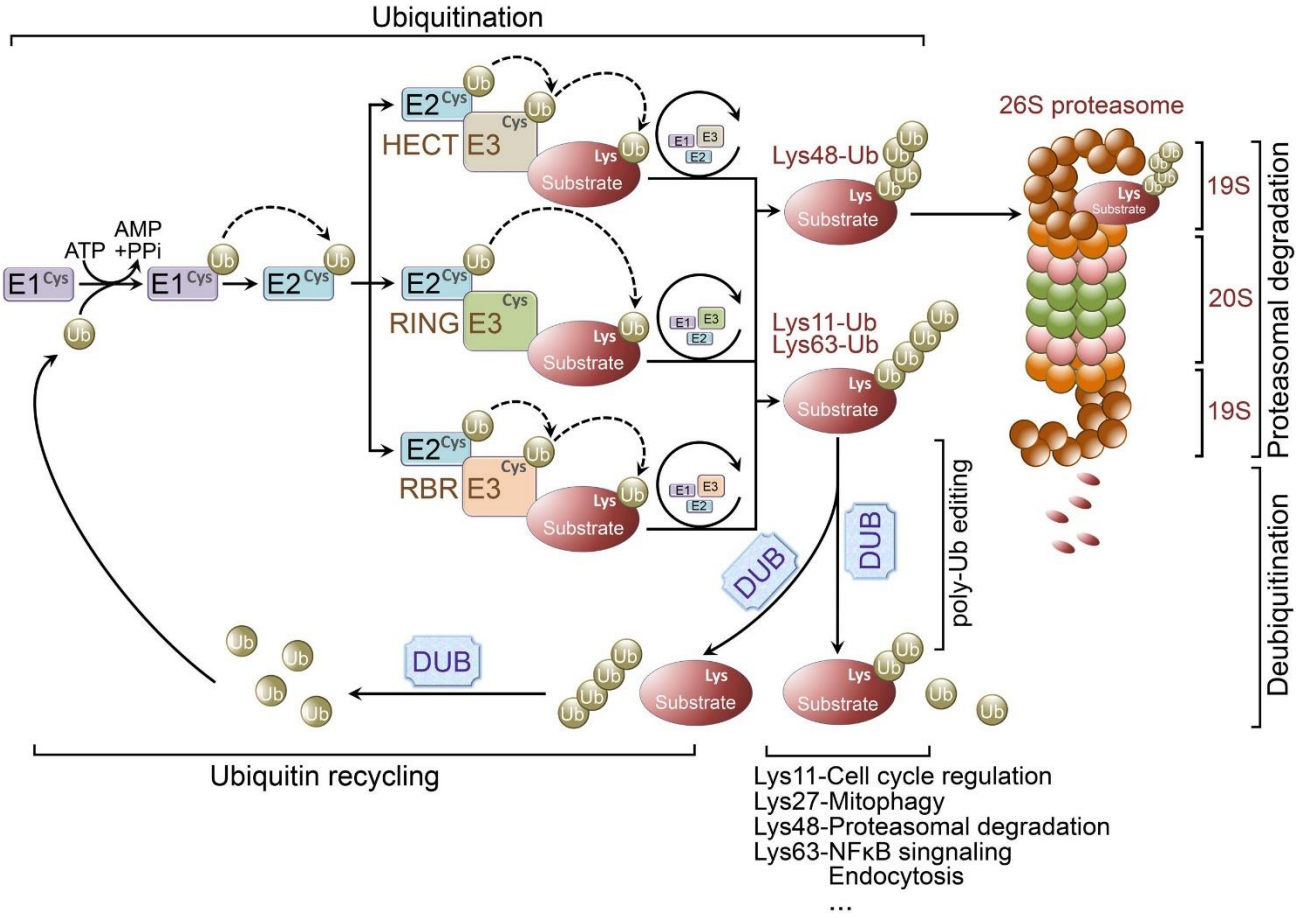



**Figure 1.2: The Ubiquitination pathway.** The pathway begins as E1 binds and activates Ubiquitin (Ub) which is then transferred to E2. Next, the E3 ligase catalyzes the transfer of Ub to the substrates. HECT as well as RBR E3 ligase bind to the Ub itself prior to ligation with the substrate, while the RING domain would bring E2 along with the substrate in close proximity with Ub and transfer the Ub onto the substrate. Ubiquitination on different lysine residue leads to different fates of the protein. For example, K48 linked polyubiquitination results in proteasomal degradation, K63 and K11 linked polyubiquitination are involved in cellular signaling. Proteasomal degradation is carried out by 26S proteasome. Deubiquitination also occurs by deubiquitinating enzymes which cleave ubiquitin attached to substrates and rescue substrate proteins from degradation. Figure retrieved from Zheng et. al, 2016.

## 1.2 Cullin- RING E3 ligases: Cul3- BTB

As mentioned previously, E3 ligases are divided into several classes. Cullin- RING ligases (CRLs) are the largest subclass of E3 ligases. CRLs consist of multi-subunit enzymes including an exchangeable substrate adaptor bound to a cullin (Cul) based catalytic core. The interaction between the Cul and RING domain creates its catalytic core, whose main function is to recognize E2s and mediate ubiquitination (Petroski et. al, 2005). RING domain proteins are members of the zinc finger domain family of proteins. They contain a consensus sequence CX2CX [9-39]CX[1-3]HX[2-3]C/HX2CX[4-48]CX2C (C: Cysteine, H: Histidine, X: any amino acid), which are responsible for the mediation of two zinc ions (Joazeiro et. al, 2000; Freemont, 2000). The zinc finger domain interacts with E2 as well as destabilizes the thioester bond between E2 and the attached Ub, which facilitates the transfer of Ub to E3, then to the attached substrates (Das et. al, 2013). The specificity of CRL's activity is dependent on their unique substrate- binding adaptor proteins in each E3 ligase complex. (Geersdaele et. al, 2013). To date, there is an estimate of 336 RING and U- box E3s identified in the human genome, which is around 90% of all E3 ligases (Medvar et. al, 2016).

The most well- studied CRL is the Cul1-Skp1- F-box (SCF) ubiquitin ligase, which is involved in regulation of cell cycle progression through ubiquitin mediated proteolysis (Bai et. al, 1996). A Cullin type protein scaffold, in this case Cul1, serves as a connection between the E2 docking motif and substrate interacting motif. Skp1 (S phase kinase associated protein 1), which serve as the adaptors that bind to Cul1 at the N- terminus and to F-box protein through the F-box motif (Schulman et al., 2000) **(Figure 1.3 A)**. The F-box protein is also involved in substrate recruitment and binding to the complex, the

specificity of substrate binding is determined through types of F box, to date, there are 69 F- box proteins identified (Gorelik et. al, 2016). Similarly, in Cul2 based E3 ligases, the ECS complex also consists of a BTB domain containing protein Elongin B and C bound to the N- terminal of Cul2 and a substrate binding adapter SOCS- box protein (**Figure 1.3 B**).

Cullin- 3 (Cul3) RING- box- based E3 ubiquitin ligase (CRL3) contains an adapter protein bearing both the BTB domain and the substrate binding domain (Pintard et. al 2004). The BTB domain, which is the homologous region to Broad- complex Tramtrack and Bric a brac, or the POZ domain, is a highly versatile domain which mediates protein-protein interactions. The N- terminal domain of Cul3 binds to BTB, while the C- terminal domain binds to RING based E3s, which completes the CRLs. The Cul3 is a helical stalk-like scaffold, whose interaction with the RING- E3 enables the recruitment of Ub- E2s, whose interaction with BTB containing proteins recruit the substrate (Xu et. al 2003; Pintard et. al, 2004) (**Figure 1.3 C**). Many BTB proteins play important roles in transcriptional regulation, which alters gene expression through mediating chromatin conformation. One example is the human promyelocytic leukemia zinc finger (PLZF) protein, whose BTB domain negatively regulates transcription through interacting with subunits of the histone deacetylase complex. The structure of the BTB domain reveals the formation of homodimers, which is enabled by its evolutionary conserved N- terminal extensions, which consists of an additional β-strand and α-helix (Ahmad, 1998). The dimer consists largely of a hydrophobic interface, with a groove exposed on the surface for peptide- binding.

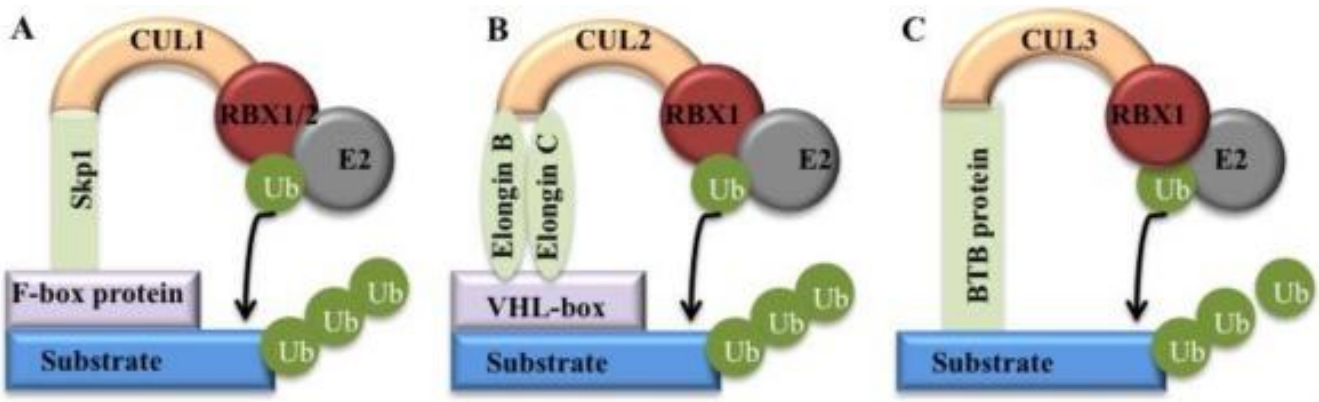



**Figure 1.3 composition of Cul1, Cul2 and Cul3 based RING ubiquitin E3 ligases.** (A) SCF, a Cul1- RING E3 ligase, consists of Cul1 proteins binding to the N' terminal of BTB protein (Skp1), and F-box protein for substrate recognition and binding. (B) ECS complex is a Cul2 based RING E3 ligase, the interaction between the BTB protein Elongin B, C with SOCS box protein recruits the substrates. (C) For Cul3 based RING E3 ligase consist of one protein which contains both the BTB domain and substrate recognition and binding domain. Figure retrieved from Chen *et. al,* 2015.

Overall, CRL3 complexes have been shown to have a variety of functions controlling different protein- protein interaction networks (Papizan et. al, 2018; McEvoy et. al, 2007). This suggests that identification of novel Cul3 substrates can lead to a better understanding of the regulatory mechanisms of different pathways. In turn, cancerous mutations occurring in subunits of CRL3 could be better understood by examining the effects they have on regulating substrate proteins. In the future, anti- cancer therapy can be developed to target CRL3- substrate protein interactions.

## 1.3 Ubiquitination in Cancer

Post-translational modifications have been found to play a critical role in promoting tumor cell proliferation, malignant progression and even drug resistance (Lu et. al, 2016). Alterations through ubiquitination contribute to cancer initiation and progression by deregulating tumor suppressors and/or up-regulating oncogenes. Previous research characterizing the ubiquitin landscape with cancerous mutations showed that mutations can occur in all E3 ligase components (Theurillat et. al, 2014), which suggests that all ubiquitin enzymes can be targets for developing cancer treatments. Previous reports showed that silencing E1 activators results in leukemia and myeloma cell apoptosis (Xu et.al, 2010); Dysregulations in E2 conjugates and E3 ligase can promote tumor growth and metastasis; various mutations in E3 ligases supports tumor cell proliferation. While mutations in E2s are mainly due to over- expression resulting in cancer cell metastasis, cancerous mutations in E3s usually lead to down-regulation of E3 (Gallo et. al, 2017).

## 1.4 Speckle - type POZ protein (SPOP)

Speckle type POZ (pox virus and zinc finger protein) protein (SPOP) was discovered and named for the nuclear speckles formed as well as homology to the Poz domain (Nagai et. al, 1997). SPOP is the adapter protein in the Cul3- RING E3 ubiquitin ligase complex (CRL3). It is responsible for recognition and interaction with substrate proteins (Kwon et. al, 2006). The main function of the Cul3- RING- SPOP E3 ligase complex is to mediate ubiquitination and target proteins for degradation. Identified SPOP substrate proteins include many oncoproteins and tumor promoters, which suggests that SPOP has anti- tumor properties (Li et. al, 2011). SPOP gained research attention due to

the high level of mutations in different cancer types, particularly in prostate cancer. This associates SPOP mutations with cancer initiation and progression. Overall, SPOP has been shown to have direct involvements in degradation of oncoproteins, mediation of the DNA damage response and other cellular pathways involved in cancer suppression (Nakayama et. al, 2006; Wei et. al, 2018).

### 1.4.1 SPOP in prostate cancer

Prostate cancer is the second most common cancer in men worldwide; it results in over 250,000 deaths each year (Jemal et. al, 2011). Common prostate cancer mutations include loss of function of NKX3.1 (Bhatia- Gaur et. al, 1999), PTEN (Li et. al, 1997), and upregulation of AR (Linja et. al, 2004) as well as ETS-family fusion proteins (Visakoripi et. al, 1995; Perner et. al, 2006). SPOP has been identified to be one of the most commonly mutated genes in prostate cancer (**figure 1.4**), exome sequencing showed that SPOP mutations are found in 11- 13% of prostate tumors (Barbieri et. al, 2012). Heterozygous SPOP substitutions were identified in 6-13% of the 300 primary prostate tumors sequenced and were not found in any of the benign prostate tissue. Recent studies analyzed the ubiquitin landscape, the ubiquitylome, revealing that mutations in SPOP can cause a shift in the ubiquitylome that promotes prostate cancer growth and development. Results from the ubiquitylome analysis showed that prostate cancer associated SPOP mutants impair ubiquitination in its substrates in a dominant- negative manner (Theurillat et. al, 2014).

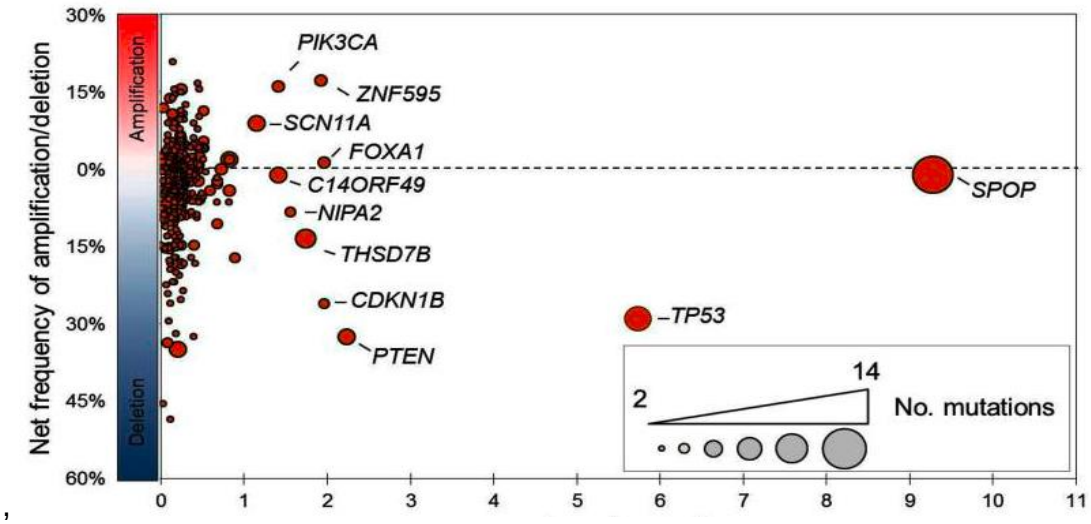



**Figure 1.4 Mutation frequencies in prostate cancer:** Net frequency of mutation in tumor samples. SPOP is identified as one of the most often mutated (autosomal genes with two or more mutations) among the 112 prostate tumors exomes sequenced (Barbieri *et al.* 2012).

In particular, prostate cancer mutations occur in the substrate binding domain of SPOP, suggesting that this mutation is positively selected for during tumorigenesis to alter substrate interaction and ubiquitination in a pro-tumorigenic manner. The most commonly mutated residues are the essential residues for substrate binding, namely Y87, W131 and most common of all, F133 **(figure 1.5)**. *In vitro* studies revealed that mutations in these locations disrupt certain substrate binding abilities (Zhuang et. al, 2009). In addition, SPOP mutations occur in a heterozygous state which could repress wild-type SPOP function by dimerization, which results in significant loss of function.

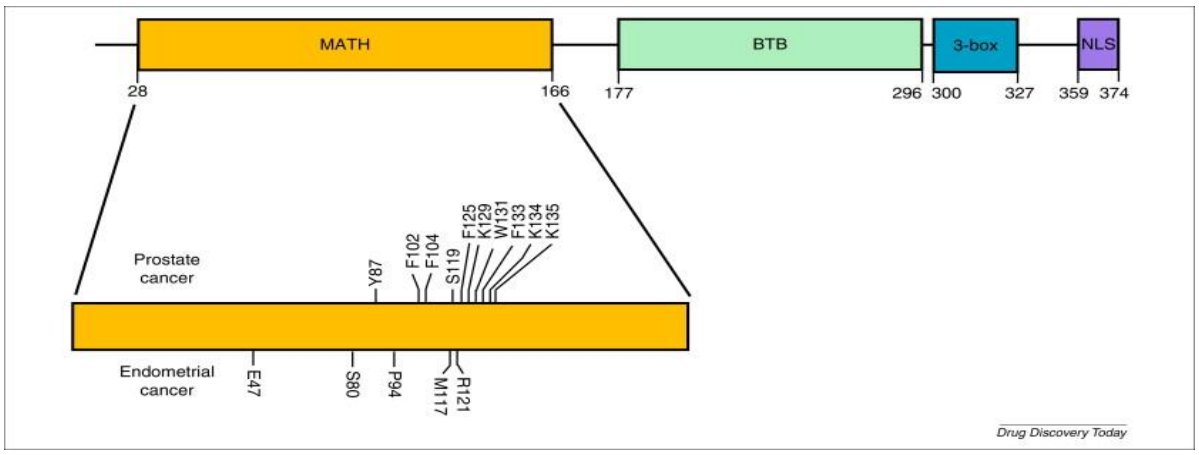



**Figure 1.5 SPOP domains and cancer mutations.** SPOP consists of three main domains, an N-terminal MATH domain, BTB domain and a C- terminal nuclear localization signaling. All identified cancerous mutations have been found to be located within the MATH domain; The most common mutations include F133V and W131G. (Mani, 2014)

Upon SPOP mutation, substrate proteins such as DEK and TRIM24 are consistently upregulated as a result of decreased levels of ubiquitination and proteasomal degradation. DEK is an oncoprotein that aids the proliferation of prostate cancer development through promoting prostate epithelial cell invasion. DEK is shown to accumulate in SPOP mutant samples, which confirms that prostate cancer SPOP mutants have impaired DEK ubiquitination and proteasomal degradation (Theurillat et. al, 2014). TRIM24 is also a key prostate cancer driver and an identified oncogenic transcriptional activator in prostate cancer which mediates activation of androgen receptor (AR) proteins and degradation of TP53 (Groner et. al, 2016). Upon wildtype SPOP overexpression, TRIM24 is down- regulated along with other known SPOP substrates. Similar to DEK,

TRIM24 also up- regulated SPOP mutant expression, especially the nuclear expression (Theurillat et. al, 2014).

Another prostate cancer associated protein target of SPOP is SRC3, which is also an AR protein. In cancer samples, mutation of SPOP results in loss of SRC3 regulation and loss of androgen receptor activity (Byun *et. al,* 2008). Other substrates of SPOP include ERG an oncoprotein, which upon mutation of SPOP leads to an accumulation of ERG protein level and leads to tumor progression. Other substrates include oncoproteins such as CDC 20, which is responsible for substrate recruitment to the Anaphase Promoting Complex leading to tumorigenesis (Wu et. al, 2017; Peters et. al, 2006); EgIN2, which facilitates prostate cancer cell proliferation (Zhang et. al, 2017); inF2, which mediates mitochondrial fission upon SPOP mutation (Jin et. al, 2017); Senp7, which promotes cellular senescence upon degradation (Zhu et. al, 2015); DDIT3, an apoptosis execution trigger from the endoplasmic reticulum stress response pathway (Zhang et. al, 2014); SETD2, involved in methylation of H3K36 important for genomic stability (Zhu et. al, 2015) and C-Myc, a potent oncogene involved in tumor cell proliferation (Geng et. al, 2017). Overall, SPOP mutations are implicating a large overlapping route of transformation in prostate cancer involving the dysregulation of multiple oncoproteins in various pathways (Wei et. al, 2018).

Mouse models have been used to demonstrate that SPOP mutations drive prostate tumorigenesis *in vivo*. Expression of mutant SPOP showed dramatic alteration in prostate organoids including early neoplastic lesions and invasive, poorly differentiated carcinoma (Blattner et. al, 2017). In the SPOP- F133V mouse model, only mice with loss of PTEN expression showed high- grade prostatic neoplasia. PTEN deletions as well as

mutations are very frequent in advanced prostate cancer and are shown in the mouse model to contribute to progression of SPOP mutant prostate cancer (Haffner et. al, 2013). In addition, downstream effects of SPOP mutation showed upregulation of DEK and SCR3 as previously identified, however, AR as well as PTEN levels did not change, suggesting that SPOP mutations alone are sufficient for cancer phenotypes.

### 1.4.2 SPOP links to other cancers

Besides prostate cancer, SPOP mutations lead to altered expression of many proteins associated with cancer formation as well as progression, as seen in breast cancer, gastric cancer, renal cancer and glioma (Wei et. al, 2018). SPOP mutations and alteration of SPOP expression have been analyzed in many cancer types. Three major somatic missense mutations have been identified in multiple cancer types, S14L, W87C and F133V/L. Through investigating the transcription levels of SPOP mutants, it was found that the F133V is also the mutant that leads to the enrichment in gene expression of genes associated with BRCA1 mutations commonly found in ovarian and breast cancer.

Sequencing studies revealed that SPOP mutations also occur in endometrial cancer but on different residues from prostate cancer. In multiple types of cancer, SPOP mutation leads to poor prognosis due to its function as a crucial regulator of progesterone protein levels (Tan et. al, 2017). Other SPOP substrates such as HDAC6, MMP2 and SIRT2 are associated with gastric cancer, lung cancer and colorectal cancer (Kim et. al, 2013). Taken together, SPOP is a powerful tumor suppressor in diverse cancer tissue types. However, in some cases, SPOP also acts as a tumor promoter. By mediating degradation of Daxx, Gli2, PTEN and DUSP7, tumorigenesis occurs and inhibition of

SPOP binding to substrates promoted renal cancer cell death (Guo et. al, 2016). Overall, dysfunctional SPOP is highly associated with many types of cancer and loss of SPOP function drives tumorigenesis through many different pathways.

However, not all cancer types show downregulation of SPOP. In renal cancer, specifically clear cell renal cell carcinoma, SPOP is shown to be overexpressed in the cytoplasm. Since SPOP is normally localized in the nucleus, misallocation to the cytoplasm causes a significant decrease in cytoplasmic regulatory proteins for cellular proliferation and apoptosis including tumor suppressors, which creates an optimal environment for tumorigenesis and tumor cell proliferation (Li et. al, 2014). Recent research showed success in inhibition of SPOP with small molecule inhibitors which inhibited SPOP- mediated ubiquitination. Targeting SPOP for cancer treatment is ideal as it is tumor specific and a small molecule inhibitor disrupts degradation of tumor suppressive SPOP substrates, which leads to cancer cell death (Guo et. al, 2016).

### 1.4.3 SPOP in DNA Damage response

Evidence also suggests that SPOP is able to affect double stranded DNA damage and repair pathways by blocking homologous recombination (HR) repair. SPOP has been shown to interact with ATM, a critical DNA damage response (DDR) protein which initiates the DDR pathway. After a double- stranded DNA break is detected, SPOP co- localizes with yH2AX foci (Zhang et. al, 2014), and favors non- homologous end joining (NHEJ) for repair which is more error prone compared to HR (Boysen et. al, 2015). Prostate cancer mutation of SPOP results in a high degree of genomic instability and a lack of HR via dysregulation of key DNA repair factors including BRCA1, ATR, CHK1 and RAD51

(Hjorth- Jensen et. al, 2018). Silencing of SPOP causes altered CHK1 levels and results in decreased levels of all of the above mentioned proteins. SPOP has been shown to impair RAD51 foci formation, which is important to overcome replication fork stalling and respond to DNA double stranded breaks. Loss of SPOP function also leads to increased 53BP1 bodies and micronuclei formation in G1 cells, which are signs of spontaneous replication stress as well as genomic instability (Hjorth- Jensen et. al, 2018).

### 1.4.4 SPOP Structure Overview

SPOP consists of three domains: A N-terminal MATH domain (28-166) for substrate recognition and binding; an internal BTB domain (190-297) which mediates interaction with cullin-3 and a C- terminal end which consists of a nuclear localization sequence (365-374) **(Figure 1.3)**. SPOP dimerizes and binds Cul3 through its BTB domain, the crystal structure of the BTB domain reveals dimerization of 2 BTB:2 Cul3 complex. Residues 177- 297 of the BTB are dimerized through its hydrophobic interface, which is a shared feature across the BTB family, similar to that of Spk1 and Cul1 (Xu et. al, 2002).

#### 1.4.4.1 MATH domain

The MATH domain of SPOP comprises of an anti- parallel β- sandwich which has high structural superimposition with most MATH and TRAF domains. The MATH domain is an asymmetrical dimer located in the middle of the V shaped groove by the BTB domain, which is a dimeric domain that binds to Cul3 (**Figure 1.6**). The dimeric BTB domain connects with two Cul3, therefore creating a complex comprised of two substrate binding sites and two catalytic cores. The dimeric structure enables high levels of flexibility in

substrate binding and elongating ubiquitin chains. Mutations in the SPOP MATH domain reduce its ability to interact with its substrates resulting in dominant negative effects in substrate binding, ubiquitination and degradation (Theurillat et. al, 2014).

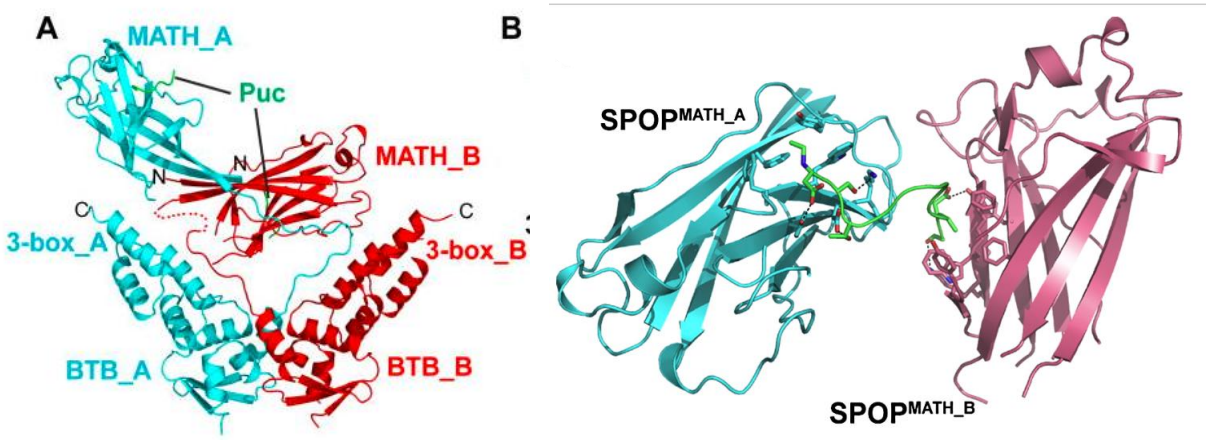



**Figure 1.6**. Structures of SPOP MATH domain and SPOP BTB domain. (A) Overall structure of the SPOP MATH -BTB dimer, crystal structure obtained by Zhuang *et. al* 2009, with one molecule in blue and a second one in red. (B) Two isolated MATH domains binding to a single substrate is shown in green, substrate binding occurs in the groove in between the two monomers.

#### 1.4.4.2 SPOP Binding Consensus

The SPOP Binding Consensus (SBC) motif is identified as φ-π-S-S/T-S/T (φ-nonpolar; π-polar). The MATH domain is the Meprin and TRAF homology domain, which binds selectively by interacting with the serine/threonine - rich degrons in target substrates. It was shown that the interactions and biological functions *in vivo* depend

critically on cooperativity among the S/T rich domain to achieve high occupancy (Zhuang et. al, 2009). Intriguingly, all the mutations in prostate cancers are localized in this domain, altering its substrate binding abilities (Barbieri et, al, 2012).

The SBC motifs are comprised of extended conformations which bind to the shallow groove of the MATH domain DWGF. All of the SPOP substrates identified thus far contain the identical binding consensus and similar binding affinities. The interactions are affiliated by both polar and hydrophobic interactions. In particular, F102, W131 and F133 are responsible for interaction with the hydrophobic side chains at position 1, which encloses the side chain in the shallow groove (K129, D130 and W131). While residues D130 and Y87 are responsible for water mediated hydrogen- bond formation with the serine or threonine rich side chains. Serine at position 3 of the motif is highly important for substrate binding as it is both able to form hydrogen bonds and is constrained by size to fit in the shallow groove. Mutation studies revealed that residues D130 and W131 are crucial in substrate binding, mutations D130A and W131A were highly deleterious when the interaction was disrupted *in vitro* (Zhuang et. al, 2009).

#### 1.4.4.3 MATH -BTB

SPOP dimerizes and binds Cul3 through its BTB domain. The structure of SPOP BTB domain revealed a hydrophobic interface for its dimerization consistent with the structure of Cul1, Cul2 and Cul5; the BTB binds to Cul3 in the same manner as Skp1 and Cul1 (Xu et. al, 2003). In addition, the BTB domain also contains a pair of C- terminal α-helices. In other Cullin binding complexes, the helices are important to physically link the BTB- like- fold adaptors to substrates binding domains. In the BTB, the pair of helices are

at the distal end from the site that binds to the MATH domain, suggesting secondary functions of BTB protein. However, the exact sequence recognition for Cul3 remains unclear.

Looking at the structure of MATH and BTB domain together, the two domains form an asymmetric dimer with a difference in orientation by 55°. The MATH domain is located in the middle of the V- shaped groove of the dimeric BTB domain, while the other MATH domain is extended away from the BTB domain, however it also in the shape of a V. The two domains are highly flexible, there is minimal contact between the MATH and BTB, and the linker is not visible in certain structures. This flexibility allows the substrate binding site of MATH domains to bind to more structurally diverse substrates as well as to increase binding affinity, as there are two MATH domains to be utilized and many substrates contains more than one binding sequence. Dimerization also plays a role in ubiquitination as dimerization defective SPOP is not capable to ubiquitinate its substrate under any condition. Overall, SPOP consists of an asymmetrical dimmer with two highly flexible substrate binding sites and a flexible Cul3- RING core. This allows SPOP to have a large range of conformations to recognize a large variety of substrates and to mediate ubiquitination in multiple ways (Zhuang et. al, 2009).

### 1.4.5 Other Known SPOP substrate

SPOP substrates are involved in a variety of essential cellular functions (Mani, 2014). Known substrates of SPOP include DAXX, an apoptotic regulator which showed reversed repression of p53- dependent transcription upon SPOP expression (Kwon et. al, 2006). DAXX is known to interact with ETS-1 to repress transcriptional activation of ETS-

1 (Li et. al, 2000). The degradation of DAXX by SPOP will result in reversed transcriptional activation of EST-1. Sequence reveals that DAXX carries the SPOP binding motif VSSTT (Kwon et. al, 2006). Results also showed that this interacts specifically to the MATH domain and upon deletion of the MATH domain, this interaction is blocked. It was also shown that this process is proteasomal dependent, as upon inhibition of the proteasome the Cul3- SPOP mediated DAXX degradation is reduced. Consequently, knockdown of SPOP and CUL3 resulted in upregulation of DAXX levels. This complex is a crucial regulator of Vascular endothelial cell growth factor receptor 2 (VEGFR2), which is an essential receptor for endothelial cell homeostasis, mainly angiogenesis and can lead to the development of angiogenic therapies (Sakaue et. al, 2017). Taken together, these results indicate that SPOP plays an essential role in recruiting DAXX through its MATH domain to Cul3- based Ub ligase for ubiquitination of DAXX and its subsequent proteasomal degradation.

The SPOP MATH domain is also known to interact with BMI1, a PRC1 protein in the polycomb group (PcG) proteins to impose gene repression by initiating X chromosome inactivation for epigenetic gene regulation (Hernández-Muñoz 2005). SPOP mediates ubiquitylation of BMI1 and allows the re- localization of MacroH2A1 to the inactive X chromosome (Takahashi et. al, 2002).

PTEN, phosphatase and tensin homolog, is a known tumor suppressor and is also a SPOP substrate. Since down- regulation of PTEN occurs in many cancer types, it was shown that tumorigenic activities are the result of SPOP mediated degradation of PTEN resulting in altered cell proliferation levels (Li et. al, 2014). In cases like kidney cancer, overexpression of SPOP was found in 85% of patients, therefore resulting in significant

decrease in PTEN levels in kidney cancer tumors (Liu et. al, 2009). Structural studies revealed that SPOP MATH domain interacts with PTEN SBC residues ASSST and mutations within this motif showed reduced degradation of PTEN (Li et. al, 2014). However, the regulation of SPOP activity is not mediated through its MATH domain. Unlike kidney cancer, up to 15% of prostate cancer showed inactivated SPOP, which also correlates with PTEN-deficiency in prostate cancer cells.

### 1.5 Another MATH/TRAF domain protein – USP7

An important protein in the ubiquitination pathway which contains a MATH domain is Ubiquitin specific protease 7 (USP7). USP7 is an important deubiquitinating enzyme in mammalian cells that has been implicated in many cellular functions including DNA damage as well as repair, transcriptional regulation and is found to be highly implicated in cancer development. USP7 consists of 3 domains: A C- terminus domain made up of five ubiquitin like motifs, a catalytic core and an N' terminal tumor necrosis factor -receptor associated factor (TRAF) or MATH domain. The MATH domain is also known to function as a substrate recognition and binding domain. Many substrates of USP7 have been identified including tumor suppressors p53 and MDM2, as well as Ubiquitin- conjugating enzyme E2E1 (UbE2E1), ubiquitin- conjugating enzyme E2E2 (UbE2E2) and ubiquitin-conjugating enzyme E2E3 (UbE2E3) (Sheng et. al, 2006; Sarkari et. al, 2013, Davarinejad and Ashurov, unpublished).

The best characterized role of USP7 in cancer is its regulation of p53- murine double minute protein (MDM2) - MDMX pathway. p53 as a tumor suppressor binds to the MATH domain of USP7 which regulates its stability via deubiquitination, which in turn

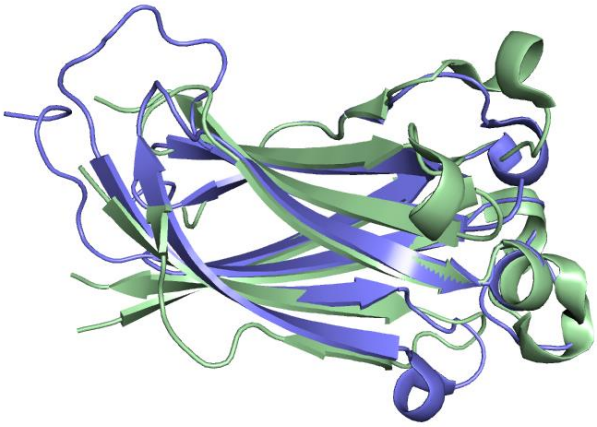
rescues p53 from degradation (Li et. al, 2002). In addition, USP7 stabilizes MDM2, which is responsible for the ubiquitination of p53 (Li et. al, 2004; Cummins et. al, 2004). USP7 plays an important role in regulating both of p53 and MDM2 levels in order to maintain p53 at an optimal level. Moreover, USP7 also has a large subset of viral binding partners which competitively bind to USP7 at the same site as its cellular substrates. In multiple cases it's been shown that many viruses exploit this interaction site in order to hijack USP7, which downregulates p53 as well as promotes cell cycle progression, thus optimizing viral protein expression and in some cases, leading to tumorigenesis along with cancer (Everett et. al, 1997; Lee et. al, 2009; Saridakis et. al, 2005). Viral proteins from Epstein- Barr virus (EBV), human cytomegalovirus (HCMV) and herpes simplex virus (HSV) have all been shown to bind to USP7. (Sivachandran et. al, 2008; Holowaty et. al, 2004; Chavoshi et.al, 2016; Salsman et. al, 2012; Bhattacharya et. al, 2018)

The USP7 substrate binding site is identified as $^{164}$DWGF$^{167}$ in its MATH domain and forms a shallow groove for substrates to bind (Sheng et. al, 2006) and that mutations in these residues led to the loss of binding between the substrates and USP7. The USP7 binding motif was identified to be P/A/ExxS (Sheng et. al, 2006; Hu et. al, 2006), in which X stands for any amino acids. In particular, cellular substrates of USP7 contain the preferred (P/A) STS motif, while the viral proteins generally adapt the EGPS motifs.

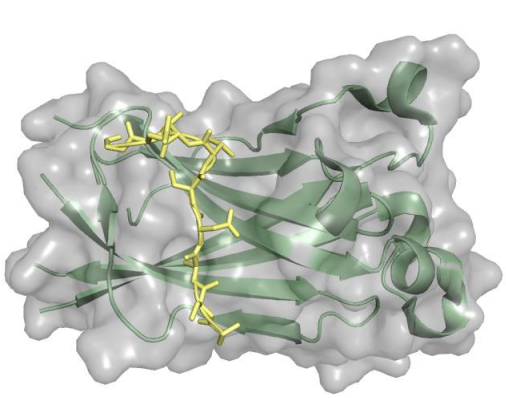
## 2. PROJECT RATIONALE

Knowing the clinical significance of SPOP and that mutations in the MATH domain are responsible for cancer initiation, it is important to understand the interaction of the MATH domain with its substrates. This project aims to identify novel SPOP interacting proteins as well as to investigate whether these proteins are bona fide Cul3- SPOP substrates. Due to the high structure (**figure 2.1**), sequence (**figure 2.2**) and binding motif similarity to the MATH domain of USP7, we hypothesize that SPOP should be able to interact with a large number of USP7 substrates through the DWGF motif.

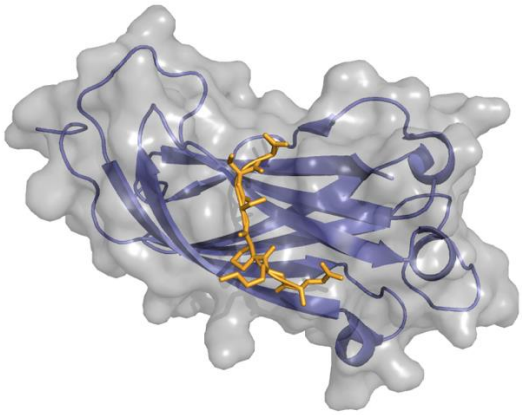
There are already a considerable number of overlapping substrates of SPOP and USP7, including DAXX, BMI1, C- Myc, and PTEN. This provides a basis for selecting potential SPOP substrates by starting with known USP7 substrates containing the φ-π-S-S/T-S/T sequence motif. USP7 interacts with several proteins through its MATH domain containing the E/P/AxxS motif. DAXX is a known USP7 substrate (Tang et. al, 2010), although the exact binding sites of DAXX is not confirmed, Eukaryotic Linear Motif (Gouw et. al, 2017) identifies many sequences matching the binding motif of USP7, which includes the SPOP binding sites ADSST and VSSTS. In addition, E/P/AxxS sequence is a well- established USP7 MATH domain binding consensus. If SPOP and USP7 indeed share a large subset of substrates, substrates harboring the E/P/AxxS sequence are likely SPOP substrates as well. The most common binding consensus is EGPS, especially with the viral substrates of USP7. This also suggests the possibility that substrates containing the EGPS motif are also potential substrates of SPOP.



(a)



(b)



(c)

**Figure 2.1 (a)** Structural similarity of SPOP MATH domain and USP7 MATH domain. Structural alignment of the SPOP MATH domain is represented with a green ribbon and the USP7 MATH domain is shown in blue. Structures retrieved from RCSB protein data bank, and figure generated using PyMOL (Schrödinger 2010). **(b)** Structure of SPOP-MATH domain interacting with MacroH2A peptide (Zhuang et. al, 2009). Structure retrieved from RCSB protein data bank (PDB: 3HQH), figure generated using PyMOL. **(c)** Structure of USP7- MATH domain interacting with MDM2 peptide (Sheng et.al, 2006). Structure retrieved from RCSB data bank (PDB: 2FOP), figure generated using PyMOL.

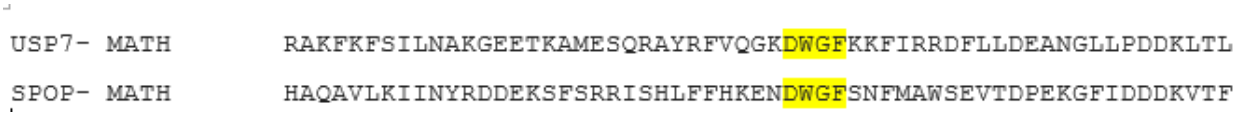



**Figure 2.2** Sequence alignment of MATH domain of USP7 and SPOP. Sequence retrieved and aligned from UniProt (The UniProt Consortium). The alignment provides evidence for sequence motif similarity. Most prominent features include the DWGF motif.

This study aims to discover new cellular substrates and viral binding partners of SPOP in order to investigate the involvement of SPOP in previously unknown cellular pathways. We also aim to see how SPOP levels affect stability of the identified binding partners. Lastly, we want to see the effect of the prostate cancer SPOP mutation, F133V, in its substrate binding. This would provide new insights in understanding the molecular mechanism of tumorigenesis caused by SPOP mutation, which could ultimately reveal new routes for developing novel therapeutic interventions.

**Aim 1: Discovery of novel cellular substrates of SPOP**

USP7 targets many cellular substrates involved in DNA replication, epigenetic regulation, mitosis and cell survival. Multiple studies identified protein-protein interactions involving the USP7 MATH domain, specifically DWGF, with the above-mentioned motif, E/P/AxxS. This study aims to identify new binding partners of SPOP, then investigate how SPOP regulates these identified proteins. Selected SPOP interacting candidates for this study are discussed below.

**MDM2** is an oncogenic E3 ligase for p53. It functions to keep p53 levels relatively low when cells are not under any stress. It is a highly regulated protein in DNA damage

response and is responsible for the down regulation of p53. The N terminal domain of USP7 binds to both p53 and MDM2, mutagenesis and structural data confirms the binding motif to be P/AXXS. MDM2 has the sequence PSTSS, which is identical to that of BMI1, suggesting that MDM2 is a potential substrate of SPOP (Sheng et. al, 2006).

**UbE2E1** is an E2 ubiquitin conjugating enzyme belonging to class III of E2 ubiquitin enzyme, and is known to mediate histone ubiquitination. Like all class III E2s, UBE2E1 consists of a conserved ubiquitin- conjugating (UBC) core domain (Summers et. al, 2008). It also has an N terminal extension. As a known USP7 substrate, UBE2E1's N- terminal sequence containing the ASTSS motif was shown to interact with the USP7 MATH domain (Sarkari et. al, 2013). In turn, USP7 deubiquitinates and regulates its stability (Sarkari et. al, 2013). The ASTSS motif of UBE2E1 aligns with the SPOP binding motif, suggesting that UBE2E1 is a potential SPOP substrate. **UbE2E2** and **UbE2E3** are also identified USP7 substrates and potential SPOP substrates. UbE2E2/3 also contain the N-terminal extension which harbors the P/A/ExxS motif PSTS. Structural data confirmed that USP7 can interact with E2Es and that E2Es directly associate with the USP7 TRAF domain (Davarinejad, unpublished).

**CHFR**, Checkpoint with forkhead and RING finger domains, is a mitotic stress checkpoint protein present at anaphase and regulates activities involving the E3 ubiquitin ligase activity through its RING finger domain. CHFR is involved in activating the formation of polyubiquitin chains and inducing degradation via ubiquitination (Privette et. al, 2008). In cancer and tumor cells, CHFR is often found to be mutated or silenced through promoter -methylation (Toyota et. al, 2003). Studies have shown that USP7 interacts with CHFR and greatly increases the stability of CHFR (Oh et. al, 2007). Further

structural analysis confirmed that the CHFR sequence PSTS interacts with the MATH domain of USP7 (Luthra, unpublished). This data suggests that CHFR is a potential substrate of SPOP through binding at the PSTST sequence.

**TFPT**, TCF3 fusion partner, functions in promoting apoptosis in a p53 independent manner. First identified in childhood pre-B acute lymphoblastic leukemia, overexpression of TFPT promotes caspase 9- dependent apoptosis, which contributes to leukemogenesis (Franchini et. al, 2006). Previous members of the Saridakis lab have identified an interaction between TFPT and USP7 (Luthra, unpublished). TFPT contains the known MATH domain interacting motif, EGPS, and a second binding site ALDSS, which mostly aligns with the SPOP binding consensus.

**RING1B** is an E3 ubiquitin ligase, it is mainly involved in regulating Polycomb-Group (PcG) mediated gene silencing by monoubiquitination of nucleosome histone H2A (Weake, 2008). RING1B, as the E3 component in Polycomb Repressive Complex 1 (PRC1) is known to bind to BMI1, which also relies on RING1B for its self- stabilization. RING1B is also known to undergo ubiquitination by heterologous E3, which includes E6-associated protein as a ligase (Zaaroor- Regev et. al, 2010). USP7 was also identified to regulate the ubiquitination state of RING1B and increase its stability. Through interaction of the RING domain, USP7 interacts with the same domain as the E2 recruiting and PcG protein binding domain (Bezsonova et. al, 2009). The interacting motif of RING1B with USP7 is identified as ATASG and AGPSN, which partially aligns with the SPOP sequence motif, suggesting RING1B is a possible SPOP substrate.

**Aim 2: Discovery of viral binding partners of SPOP**

USP7 is also known as herpesvirus-associated ubiquitin -specific protease. There is a significant amount of viral proteins that are known to interact with USP7. An early example is the interaction between USP7 and ICP0, a key regulatory protein in herpes virus (Everett et. al, 1997; Boutell et. al, 2005). As mentioned previously, the significance of the virus targeting USP7 is to destabilize the cellular protein which enables viral infection. The viral and cellular proteins compete for binding to the same site on USP7. This also suggests that SPOP can be targeted by these viral proteins using the EGPS interacting motif. It is possible for viral proteins to interact with SPOP, therefore this is a potential pathway for viral mediated interference in the DNA damage response and virus induced cancer. Currently, there are no known viral proteins that interact with SPOP.

Kaposi's sarcoma- associated herpesvirus (KHSV) proteins **vIRF1**, **vIRF2**, **vIRF3 and vIRF4** are proteins responsible for deregulating host cellular mechanism by weakening interferon (IFN) - mediated antiviral pathway of host cells. IRFs, viral IFN regulatory factors, are responsible for the activation of IFN pathways, which activate expression of antiviral genes upon initiation. However, viral interferon regulatory factors (vIRFs) are homologous to cellular IRFs and therefore are able to subvert the host's defense by inhibiting IRF initiation of antiviral pathways. USP7 has been identified to interact with vIRF1 as it harbors the EGPS motif and this binding decreased the level of p53 (Chavoshi et. al, 2016). Since EGPS is a potential SPOP binding sequence, vIRF1, is likely a SPOP substrate. This EGPS is also present in vIRF3 suggesting its potential interaction with SPOP and USP7. vIRF2 contains 17 potential USP7 binding sites, the two that are most aligned with the SPOP binding sequence are PTTSS and PSPSQ.

vIRF4 also contains 17 potential USP7 binding sites, the two that are most aligned with SPOP are VTTSS and PSSST.

Epstein-Barr virus nuclear antigens (EBNAs) are involved in the maintenance of viral genome in latent infection of Epstein- Barr virus (EBV). EBVs are gamma herpesvirus that have a long life- cycle which promotes a latent stage of infection (Rickinson et. al, 2001). **EBNA1** is shown to bind to its DNA sequence and recruits USP7 to the EBV genome as well as enables the downstream recruitment of GMP synthase (Frappier, 2012). The 395 - 450 regions mediate interaction with USP7 which negatively regulates replication (Holowaty et.al, 2003). This interaction also occurs through the EGPS sequence with high affinity. **EBNA2** also contains an EGPS sequence which suggests its interaction with USP7 and SPOP.

**Pp71** is a tegument protein of the human cytomegalovirus (HCMV) which is responsible for initial infection and leads to the expression of major immediate early genes, which promotes the initiation of lytic infection (Saffert et. al, 2007). Previous members of the Saridakis lab identified the interaction between pp71 and USP7 through its ASSS, EGPS as well as ASTS sequence. This interaction greatly decreases cellular levels of p53 and its downstream DNA damage associated protein (Chavoshi, unpublished). The ASSS, EGPS, and ASTS sequences align with the SPOP binding consensus, suggesting a potential interaction.

| | | φ-π-S-S/T-S/T | E/P/AxxS | USP7 binding | SPOP binding |
|---|---|---|---|---|---|
| CELLULAR PROTEIN | DAXX | AGM**VSSTS**FNG | DSG**EGPS**GMA | Yes | Yes |
| | BMI1 | GGI**PSTSS**CLP | | Yes | Yes |
| | PTEN | NPE**ASSST**SVT | | Yes | Yes |
| | MDM2 | YSQ**PSTSS**SII | | Yes | |
| | UbE2E1 | DSR**ASTSS**SSS | | Yes | |
| | UbE2E2 | | DDS**PSTS**GGS | Yes | |
| | UbE2E3 | DES**PSTSS**GSS | | | |
| | CHFR | EPQ**PSTST**SDL | GDA**PSTS**VSL | Yes | |
| | RING1B | | IYI**ATAS**GQF | | |
| | TFPT | | APG**EGPS**GRK | | |
| VIRAL PROTEIN | vIRF1 | | SPG**EGPS**GTG | Yes | |
| | vIRF2 | TPT**PTTSS**AAE | | Yes | |
| | vIRF3 | | SGD**EGPS**TRH | Yes | |
| | vIRF4 | QEE**PSSST**RQA | | Yes | |
| | EBNA1 | | EPG**EGPS**TGP | Yes | |
| | EBNA2 | | DPD**EGPS**WRP | | |
| | ORF45 | | DPD**EGPS**WRP | Yes | |
| | PP71 | | APG**EGPS**GRK | Yes | |

**Table 2.1** Protein sequence alignment of known binding partners of USP7 with known and potential binding partners of SPOP. Sequences are aligned at the **φ-π-S-S/T-S/T** motif and potential **E/P/AxxS** motif. NOTE: This is not a list of complete USP7 or SPOP substrates, if a protein contains more than one binding site, only one is shown as example.

**Aim 3: Investigate effects of prostate cancer SPOP mutations**

After identifying SPOP interacting proteins and see how SPOP is affecting them, we aim to investigate if these interactions are abolished upon SPOP mutation. In particular, we will be looking at the prostate cancer mutation, F133V. We will test the ability for the SPOP mutant to interact with previously identified binding partners.

## 3. MATERIAL AND METHOD

### 3.1 Cell Culture and antibodies:

Human osteosarcoma (U2OS) cells were used in this research. U2OS cells were grown in McCoy's medium (Wisent, 317- 010- CL) with 10% Fetal Bovine Serum (FBS) (Wisent, 080- 110) and 1mg/ml penicillin- streptomycin (ThermoFisher, 15140122). Antibodies used for immunoblotting and immunoprecipitation include anti- SPOP (Proteinteck, 16750-1-AP), anti- FLAG (Sigma, F1804; Bethyl Laboratories, A190-102A), anti- Myc (Cell Signaling Technologies, 14038), anti- HA (Santa Cruz, 7392), anti- USP7 (Millipore, 05- 1946), anti- DAXX (Cell Signaling Technology, 4533), anti- MDM2 (Cell Signaling Technology, 86934), anti- GST (Novagen, 71097-3), anti- His (Santa Cruz, 8036), anti-CHFR (Cell Signaling Technology, 6904), anti- TFPT (Sigma, HPA034958), anti- RING1B (Cell Signaling Technology, 5694), anti- UbE2E1 (BostonBiochem, A-630), anti- UbE2E2 (Bethyl Laboratories, A303- 485A), anti- UbE2E3 (Aviva System Biology, ARP43070_P050), anti- GAPDH (Santa Cruz, 47724) and normal mouse anti- IgG (Santa Cruz, 2025) antibodies. Secondary HRP- conjugated anti-mouse IgG (Jackson ImmunoResearch, 115-035-166) and anti- rabbit antibodies (Cell Signaling Technology, 7074S) were used. All antibodies were used according to manufacturer instructions.

### 3.2 Plasmids and Cloning

Full length SPOP clone (1-377) as a FLAG fusion in pcDNA3.1, (will be referred to as FLAG- SPOP) was obtained from Privé lab (University of Toronto), SPOP insertion was made with BamH1/Xho1 restriction digest site. Full length SPOP with Myc tag was cloned in pcDNA3 vector with the restriction digest sites Kpn1/Not1. C-Myc tag is encoded

in the forward primer, primers (see end of this section) were synthesized by IDT technologies and were resuspended in milliQ water to a final concentration of 10 µM. Myc- SPOP insertion was generated through polymerase chain (PCR) reaction using Phusion PCR MasterMix (NEB) with 50ng of DNA template, 10 µM of forward and reverse primers and 3% DMSO. PCR cycle is as followed: 1 cycle of 10 mins at 95°**C**, 30 cycle of 1 mins at 95°**C**, 1 min at 55°**C** and 5 mins at 72°**C**, followed by 1 cycle of 20 mins at 72°**C**. PCR product were resolved on a 1% agarose for 25 mins at 130V and purified through gel extraction (QIAgen). The insertion is the introduced to pcDNA3 mammalian vector digested with KpnI/NotI and ligated using T4 DNA ligase (NEB) at 16 °**C** overnight. The ligated DNA was transformed into E. coli chemically competent DH5$\alpha$ cells with 1 µl DNA in 50 µl cells through heat shock (10 mins on iced, 45 seconds at 42°C and 2 min on ice). Transformed cell are then selected on a 50 µg/ml ampicillin LB agar plates overnight at 37°C. Resulting colonies were cultured overnight in 5 ml of LB media with 100µg/ml ampicillin at 37°C. DNA is then extracted from the culture using the QIAprep Spin Miniprep Kit (QIAgen). Positive clone was confirmed with sequencing using CMV forward primers. Cloning primers are as follows:

Forward:

5’-GGCAGGTACCATGGAACAGAAGCTGATCTCAGAGGAGGACCTGatgtcaaggg-3’

Reverse:

5’-TATATGGCGGCCGCctcgagtcaggattgcttcaggcg-3’

HA tagged full length pp71 (HA-pp71), residues 1-559, was expressed in pcGN71 plasmid from Frappier lab (Kalejta & Shenk, 2003), and FLAG- tagged full length pp71 (FLAG- pp71) constructed was generated by previous lab member, Sara Chavoshi. FLAG tagged full length vIRF1 (FLAG- vIRF1), residue 1-449, and FLAG tagged mutant vIRF1 (FLAG- ΔvIRF1), deletion of residues $^{45}$PGEGPS$^{50}$ was generated by ACGT Corporation. FLAG tagged full length EBNA2 (FLAG- EBNA2) was kindly provided by the Frappier lab. Full list of plasmids is listed in **table 3.1** below.

**Table 3.1 DNA plasmid constructs for Mammalian Transfections.**

| Name of construct | protein | vector | tag | Sequence and mutation |
|---|---|---|---|---|
| **FLAG- SPOP** | SPOP | pcDNA3.1 | FLAG | Full length (1-377) |
| **Myc- SPOP** | SPOP | pcDNA3 | Myc | Full length (1-377) |
| **HA- pp71** | pp71 | pcGN71 | HA | Full length (1-559) |
| **FLAG- pp71** | pp71 | pcDNA3.1 | FLAG | Full length (1-559) |
| **FLAG- EBNA2** | EBNA1 | pcDNA3.1 | FLAG | Full length (1-486) |
| **FLAG- vIRF1** | vIRF1 | pcDNA3.1 | FLAG | Full length (1-449) |
| **FLAG- ΔvIRF1** | vIRF1 | pcDNA3.1 | FLAG | Full length (1-449, Δ$^{45}$PGEGPS$^{50}$) |

### 3.3 Plasmid and Expression of Recombinant Proteins:

Besides the full length SPOP construct, a SPOP MATH domain construct was also used in this study. The SPOP MATH domain (His- SPOP- MATH), residues 28 to 166, generated by Genscript, containing a N-terminal hexahistidine fusion tag expressed in a pET15b vector. A SPOP mutant construct F133V (His- SPOP- MATH mutant) was also generated by Genscript and expressed in pET15b vector. An N- terminal GST tagged EBNA1 (GST- EBNA1), residues 436- 450, was expressed from pGEX-2TK vector

(Saridakis et. al, 2005). An N' terminal GST tagged vIRF1 (GST- vIRF1) construct, residue 1- 90, and GST tagged vIRF1 mutant construct with deletion of residues $^{45}$PGEGPS$^{50}$ from residue 1-90 (GST- ΔvIRF1) were expressed from pGEX2- 2TK vector (Novagen) (Chavoshi et. al, 2016). GST tagged N- terminal of UbE2E2, residues 1- 47 (GST- UbE2E2- NT) and GST tagged N- terminal UbE2E3, residues 1- 52 (GST- UbE2E3- NT), were both expressed from pET28a vector. A full length UbE2E1 (His- UbE2E1) with N-terminal hexahistidine fusion tag was expressed from pET15b vector. All UbE2E plasmids were generated by Hossein Davarinejad. Complete list of plasmid constructs used for protein expression in bacteria are in **table 3.2** below.

**Table 3.2 Plasmid constructs for Recombinant Expression in Bacteria**

| Name of construct | protein | vector | tag | Sequence and mutation |
|---|---|---|---|---|
| **His- SPOP- MATH** | SPOP | pET15b | His | 28- 166 |
| **His- SPOP- MATH mutant** | SPOP | pET15b | His | 28- 166 (F133V) |
| **GST- EBNA1** | EBNA1 | pGEX- 2TK | GST | 436- 450 |
| **GST- vIRF1** | vIRF1 | pGEX- 2TK | GST | 1- 90 |
| **GST- ΔvIRF1** | vIRF1 | pGEX- 2TK | GST | 1- 90 (Δ$^{45}$PGEGPS$^{50}$) |
| **His- UbE2E1** | UbE2E1 | pET15b | His | Full length (1- 193) |
| **GST- UbE2E2- NT** | UbE2E2 | pET28a | GST | N- terminal (1-47) |
| **GST- UbE2E3- NT** | UbE2E3 | pET28a | GST | N- terminal (1- 52) |

### 3.4 Protein Purification:

All His tagged SPOP construct were transformed in *E. coli* BL21 mgk cells cultured in TB (Terrific broth, Bioshop) induced with 0.4mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 °C and grown for an additional 16 hours. Cells were harvested at 7446 x g for 30 min at 4 °C, resuspended in lysis buffer (50mM Tris pH 7.5, 500 or 150 mM NaCl, 5 mM Imidazole, 1x protease inhibitors cocktail (1 mM Benzamide and 0.5 mM PMSF) and 1x Protease inhibitor tablet (Roche cOmplete ULTRA Tablets)). Cell lysate is sonicated at 30% amplitude, at 10 seconds on and 15 seconds off, for a total of 4 minutes. Lysates were then cleared by centrifugation for 30 mins at 41,287 x g at 4 °C. Supernatants were then collected and incubated with nickel- nitrilotriacetic acid beads (Qiagen) for 1 hour on a nutator at 4°C. Beads were then washed extensively with wash buffer (50 mM Tris pH 7.5, 500 or 150 mM NaCl and 20 mM Imidazole). Proteins were eluted with addition of 50 mM Tris pH 7.5, 500 or 150 mM NaCl and 250 mM Imidazole.

All GST tagged proteins were expressed under the same conditions as His- tagged proteins. Cells were lysed in 1x PBS with 1x protease inhibitor cocktail (1mM Benzamide and 0.5 mM PMSF) and 1x Protease inhibitor tablet (Roche cOmplete ULTRA Tablets). The lysates were then cleared by centrifugation and the collected supernatants allowed to incubate with glutathione-Sepharose resin (GE Healthcare) for 1 hour on a nutator at 4Cº. Beads were then extensively washed with PBS and a sample of proteins was eluted with addition of 20 mM reduced glutathione (GSH) as input.

### 3.5 GST - Pull down Assay:

Prior to the experiment, Myc- SPOP was transfected into U2OS cells and lysates were collected 24 hours post transfection. Purified GST- vIRF1 and GST- ΔvIRF1 mutant were bound to pre- equilibrated glutathione- Sepharose beads separately and incubated for 1 hour on a nutator at 4ºC. A negative control was done using 20 µl of empty beads. The beads were then briefly washed with PBS and incubated with at least 500 µg of U2OS lysates overexpressing Myc- SPOP. The mixture is incubated for 2 hours at 4 C˚, then washed with 5X beads volume of PBS and a sample was eluted with 20mM reduced glutathione.

The GST- Pull down between SPOP- MATH and GST- EBNA1 was performed using purified GST- EBNA1 protein bound to glutathione beads as bait and incubated with purified His- SPOP- MATH protein as prey. A negative control was also conducted using empty glutathione beads incubated with His- SPOP- MATH protein. All incubation conditions and washing were performed as previously described. All samples were then resolved on a 4-20% gradient SDS- polyacrylamide gel (SDS- PAGE) and transferred to PVDF membranes (Millipore, IPVH00010) at 100V for an hour and blocked in 5% skim milk overnight. Membranes were then incubated with anti- His and anti- GST antibodies as described previously. Blots were developed using ECL Prime Western Blot Detection kit (GE healthcare, #RPN2232) and imaged using film.

### 3.6 FLAG - Pull down Assay:

FLAG- SPOP was transfected into U2OS cells and lysates were collected 24 hours post transfection. Anti- FLAG M2 beads (Sigma- Aldrich, A2220) were pre-equilibrated

with assay buffer and incubated with 500 µg of cell lysate for 2 hours at 4 C˚. A negative control was performed using empty M2 beads. For the FLAG SPOP- UbE2E1 pull down, after briefing washing the beads, the beads were incubated with 5 nmoles of His- UbE2E1 for 2 hours on a nutator at 4 ˚C. Samples are then washed extensively and eluted with 3X FLAG peptide, then boiled in SDS loading dye and resolved on a 4-20% gradient SDS-PAGE. Proteins were then detected through immunoblotting with anti- His and anti- FLAG antibodies.

### 3. 7 His - Pull down Assay:

His- SPOP- MATH WT and His- SPOP- MATH mutant were purified as described previously and attached to nickel- nitrilotriacetic acid beads. The His-SPOP nickel beads serve as bait in the pull down experiments. For testing interaction with endogenous proteins, U2OS cells are harvested and lysed prior to the experiment to be used as prey. At least 500 µg of U2OS cell lysates were added to the beads each and incubated for 2 hours at 4 ˚C. For testing interaction with N- terminal UbE2E2 and UbE2E3, 5 nmoles of GST- UbE2E2- NT or GST- UbE2E3- NT was purified and used as prey as previously described. The beads were washed extensively, and proteins were eluted with addition of elution buffer mentioned previously. Samples are boiled with SDS loading buffer and resolved on a 4- 20% gradient SDS- PAGE Proteins are then detected through immunoblotting and imaged as previously described.

### 3.8 Co- immunoprecipitation

For testing endogenous protein- protein interactions, whole cell lysates of U2OS cells were used. Cells are lysed in radio- immunoprecipitation (RIPA) buffer (50 mM Tris-

HCl pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 20% glycerol, 1X protease inhibitor cocktail (Roche)) followed by sonication (Branson Digital Sonifier 500 with microtip) at 10% amplitude 0.5 seconds on and 1 second off for a total of 5 seconds. Cell lysates are then cleared using centrifugation at 13,000 rpm for 12 minutes. 1mg of cleared lysates are immunoprecipitated with mouse anti- SPOP antibody and mouse-IgG as negative control. Samples are incubated overnight at 4˚C on a nutator. Followed by incubation with precleared A/G agarose beads (Santa Cruz, sc- 2003) for an hour. Immunoprecipitants were washed extensively with RIPA buffer and boiled in SDS sample buffer for 5 minutes at 100 ˚C. Samples were resolved on a 12 % SDS- PAGE and detected through immunoblotting using anti- MDM2, anti- RING1B, anti-TFPT, anti- UbE2E1, anti- UbE2E2, anti- UbE2E3 and anti- SPOP antibodies.

For testing viral protein interaction U2OS cells were co- transfected with 5 µg of Myc- SPOP and 5 µg of FLAG-pp71, FLAG- EBNA2, FLAG- vIRF1 or FLAG- ΔvRIF1 per one 15cm tissue culture plate. Transfection was performed using PolyJet transfection reagent according to manufacturer's protocol (SignaGen Laboratories). The cells were washed 6 hours post transfection and harvested 24 hours post transfection. Cells were lysed as described above. Cleared lysates are incubated with either anti-FLAG primary antibody or IgG as negative control overnight at 4˚C on a nutator. Samples are incubated with beads, washed and resolved as mentioned above. Proteins are then detected through immunoblotting with anti- FLAG for pp71, EBNA2 and vIRF1; and anti- Myc for SPOP. Blots are developed and imaged as previously described.

### 3.9 Immunoblotting

Cells were transfected with empty vector pcDNA or Myc-SPOP using Polyjet transfection reagent according to manufacturer's protocol. U2OS cells were harvested 24 hours post- transfection and lysed in RIPA buffer (50 mM Tris- HCl pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1X protease inhibitor cocktail (Roche)) and sonicated for 5 seconds at 10% amplitude. Supernatants were cleared by centrifugation and boiled in SDS loading buffer for 5 minutes at 100 ˚C and resolved on 12% SDS-PAGE. Proteins were transferred to PVDF membranes at 100V for an hour and blocked in 5% skim milk overnight. Membranes were then incubated with specific antibodies as mentioned previously. Blots were developed and imaged as previously described.

Band sizes and intensities were quantified using ImageJ (Wayne Rashand, National institute of Health, USA) measuring the pixel density. All intensities measured were normalized to GAPDH as loading control. Results are then normalized to band intensity of proteins in the control empty vector (EV) lane. Mean and standard errors were calculated for each protein (error bars represent standard errors). A paired student t- test was conducted between average normalized intensity of proteins transfected with EV to proteins transfected with Myc- SPOP. Tukey's multiple comparisons test was used comparing with 95% CI of difference. The following conventions were used: * ($p \leq 0.05$), ** ($p \leq 0.01$).

# 4. RESULTS

## 4.1 DISCOVERY OF CELLULAR SUBSTRATES

To identify potential cellular substrate proteins of SPOP, protein- protein interactions were first confirmed using a co-immunoprecipitation assay. The experiment was carried out using whole-cell lysates of U2OS cells. SPOP was immunoprecipitated with mouse anti- SPOP or mouse IgG antibodies, and predicted cellular substrates listed in **table 2.1** were identified through immunoblotting. Samples were resolved on an SDS-PAGE and proteins are identified through immunoblotting (**figure 3.1)**. The proteins that show co-immunoprecipitation with endogenous SPOP include MDM2, RING1B, TFPT, UbE2E1 and UbE2E3, which indicates that these proteins interact with SPOP. The only protein that does not show interaction with SPOP was UbE2E2, as UbE2E2 was not present in the elute fraction. To eliminate the possibility of non-specific interaction, the same experiment was conducted using mouse anti-IgG antibody instead of anti- SPOP antibody. The results from this experiment showed that none of the tested proteins were in the eluted fraction. This confirmed that these proteins did not interact non-specifically with the beads.

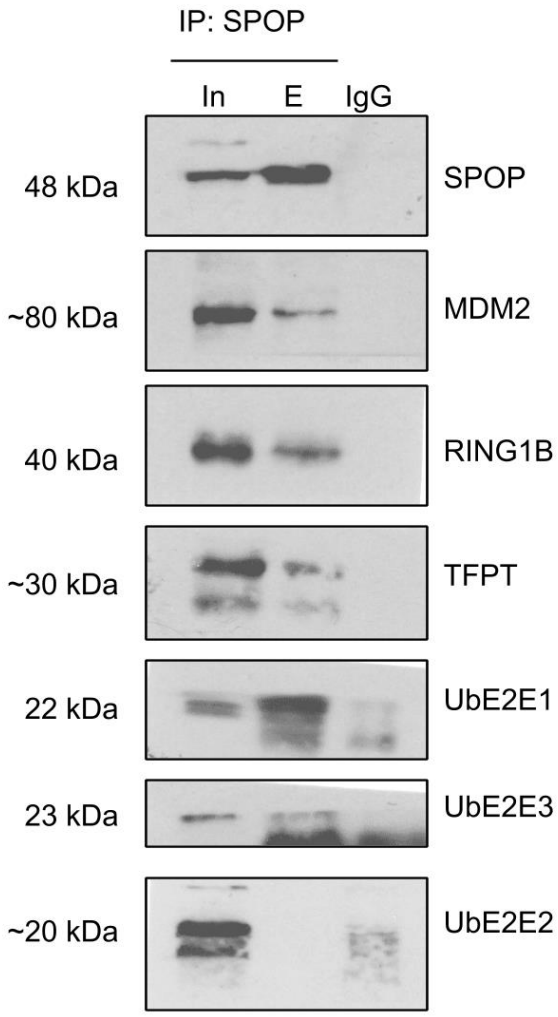



**Figure 3.1** Co- immunoprecipitation results using U2OS whole cell lysates immunoprecipitated with anti- SPOP antibody and immunoblotted for cellular proteins MDM2, RING1B, TFPT, UbE2E1, UbE2E2 and UbE2E3. 1 mg of pre- cleared cell lysates are incubated with mouse anti- SPOP or mouse- IgG as negative control, then bound to protein A/G beads. Input sample contains 5% of proteins used in experiments. Samples were resolved using a 4- 20% SDS PAGE, endogenous proteins are detected by immunoblotting with protein specific antibodies. *IP*: Immunoprecipitate*; In*, input; *E*, Elute;

### 4.1.1 PROTEIN INTERACTIONS WITH SPOP- MATH

To further investigate the interaction mechanism between SPOP and its substrates, we isolated the MATH domain of SPOP. Since the previously established DWGF binding site is located in the MATH domain, His-SPOP- MATH alone should be sufficient to use as bait for His- pull down assay. Series of His- pull downs were performed using purified His- SPOP- MATH and cellular proteins from whole cell lysates. We used recombinant His- tagged SPOP MATH domain alone protein (His- SPOP-MATH) as bait, which was bound to Nickel beads. The prey for the His- pull down experiment was obtained from whole cell lysates of U2OS cells. Samples from the inputs and eluted fractions of the pull down were resolved using SDS- PAGE and visualized using immunoblotting. The results of the His- pull down with endogenous proteins are shown in **figure 3.2**. The results indicated interaction between the SPOP MATH domain with cellular substrates including CHFR, MDM2, RING1B and TPFT as they are co- eluted in the elute fraction. A negative control was also performed using empty nickel beads incubated with the prey under the same conditions. As shown in the negative control lane, all the proteins are absent in the elute fraction, which indicates that these proteins did not interact non-specifically with the beads.

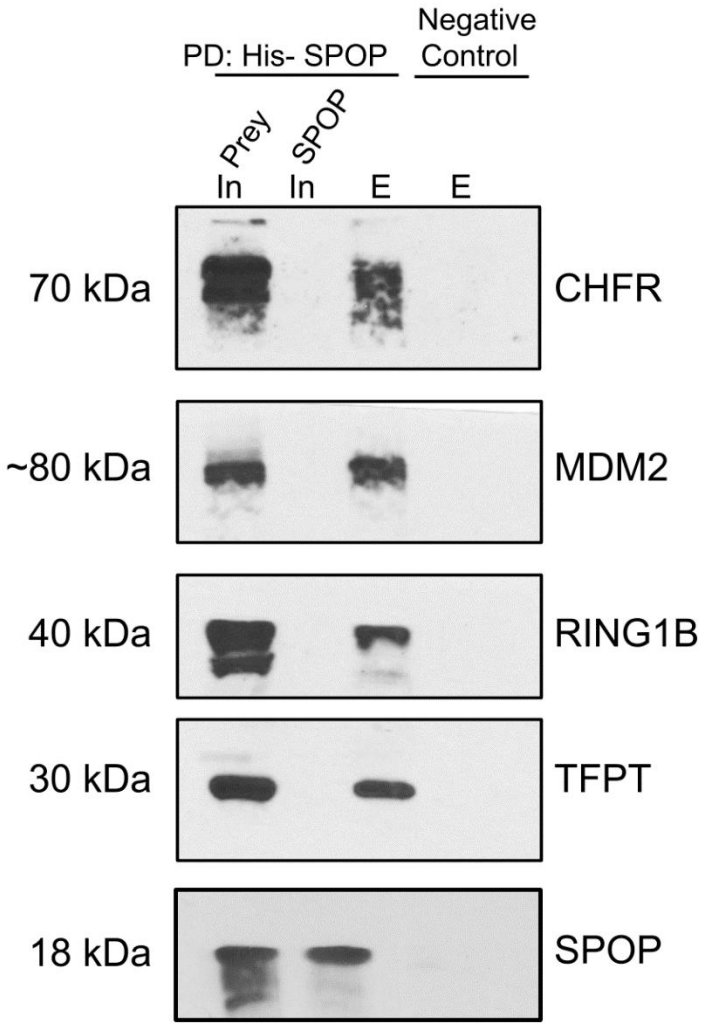



**Figure 3.2** SPOP MATH domain interacts with CHFR, MDM2, RING1B and TFPT. His-pull down experiments using purified His- SPOP- MATH bound to nickel beads as bait, incubated with 500 µg of U2OS whole cell lysates as prey. A negative control was performed using empty Nickel beads incubated with cell lysates. Input sample contains 10% of proteins used in experiments. Samples are resolved on a 4-20% gradient SDS-PAGE. The proteins are identified through immunoblotting with anti- CHFR, ant-MDM2, anti- RING1B, anti-TPFT and anti- His antibodies. *PD*: Pull down

### 4.1.2 SPOP Interacts with UbE2E1 And UbE2E3 But Not UbE2E2

The interaction between SPOP and UbE2E1 was tested using a FLAG - pull down experiment. U2OS cells were transfected with FLAG- SPOP and the cell lysate were incubated and bound to FLAG M2 beads to be used as bait. His- UbE2E1 was purified as previously described prior to the experiment and incubated with the FLAG- SPOP beads. Samples of input and elutes from the pull down were then resolved on an SDS-PAGE and UbE2E1 was identified using immunoblotting. As shown in **figure 3.3**, UbE2E1 was detected in the elute fraction, which suggests interaction with SPOP. A negative control was performed to eliminate the possibility which UbE2E1 binds to FLAG- M2 beads. Whole cell lysates of U2OS cells transfected with Myc- SPOP were incubated with FLAG- M2 beads and the pull down experiment was repeated as before. Results showed that UbE2E1 was not detected in the elute fraction without the FLAG- SPOP bound to the beads, which indicates UbE2E1 did not interact non-specially with the FLAG- M2 beads.


**Figure 3.3 UbE2E1 binds to full length SPOP**. FLAG- pull down was performed using FLAG-SPOP transfected in U2OS cells bound to FLAG- M2 beads as bait, with 5 nmols of purified recombinant His- UbE2E1 protein as prey. Samples are eluted and resolved on a 4-20% SDS- PAGE and identified using immunoblotting with anti- FLAG and anti-UbE2E1. FLAG- pull down negative control used Myc- SPOP bound to FLAG beads. Input sample contains 10% of proteins used in experiments. Proteins are identified through immunoblotting with anti-UbE2E1 and anti- FLAG.

Further experiment tested whether SPOP interacts with UbE2E2 and UbE2E3. Having shown that SPOP is able to interact with full length UbE2E3 in the Co- IP experiment, we wanted to determine if the interaction is specific between the SPOP-MATH and the N- terminal extensions of UbE2E3. As mentioned before, the class III ubiquitin E2s all consist of a conserved UBC domain along with N terminal extensions. These N- terminal domains harbor the USP7 binding sites; therefore, the N- terminal alone should be able to facilitate SPOP binding. Interaction between SPOP and N-terminal of UbE2E2 was carried out as a comparison. GST tagged N- terminal extension UbE2Es were purified using GST- affinity chromatography. His- pull downs were performed using His-SPOP-MATH bound to nickel beads as bait incubated individually with 5 nmoles of purified GST- UbE2E2- NT or GST- UbE2E3- NT as prey. Eluted samples were resolved on an SDS PAGE and proteins are identified through immunoblotting. Proteins are blotted with anti- His antibody for SPOP and anti-GST for UbE2E2 and UbE2E3. Results shown below confirmed interaction between SPOP and UbE2E3 **(figure 3.4)**. More specifically, the SPOP MATH domain interacts with the N-terminal extensions of the E2E3. Results from the pull down between SPOP and UbE2E2 suggests there is no interaction as UbE2E2 was absent in the elute fraction.

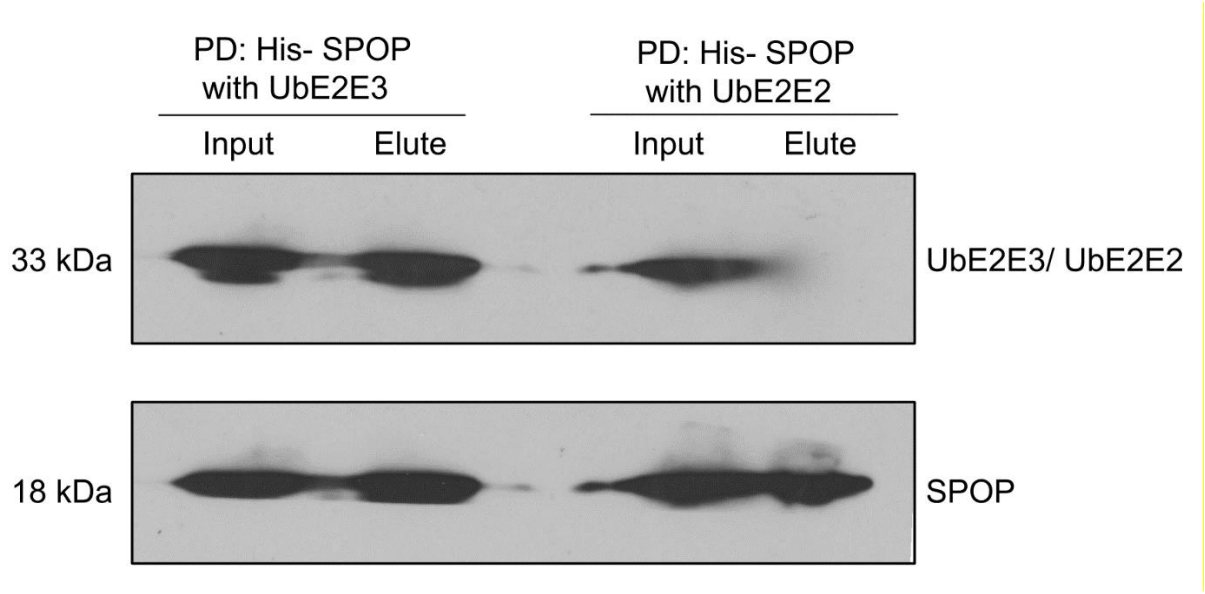



**Figure 3.4** Interactions between SPOP and the N terminal tail of UbE2E2 or UbE2E3. His- pull down was performed using His- SPOP- MATH purified and bound with Nickel-beads as bait with purified 5 nmols of GST tagged N terminal of UbE2E2 (GST-UbE2E2- NT) and UbE2E3 (GST - UbE2E3- NT) protein as prey. Input sample contains 10% of proteins used in experiments. Samples were resolved on an SDS– PAGE and UbE2E2 and UbE2E3 levels were detected through immunoblotting using anti- GST antibody, SPOP was detected using anti- His antibody.

## 4.2 SPOP's INTERACTION WITH VIRAL PROTEINS

### 4.2.1 SPOP interacts with pp71

Next, we aimed to identify novel viral protein binding partners of SPOP through series of pull down and co- IP assays. Potential binding proteins were selected from the sequence alignment list shown in **table 2.3**. We first investigated **pp71**'s interaction with SPOP. The ability of SPOP to interact with pp71 was examined using co-immunoprecipitation (co- IP). U2OS cells were co- transfected with FLAG- SPOP and HA-pp71. After immunoprecipitation of the lysate with anti- FLAG antibody, immunoblotting with anti- HA led to the identification of HA tagged pp71. A negative control was performed using mouse anti- IgG antibody which did not show any interactions. Samples are then resolved on an SDS PAGE and proteins are identified through immunoblotting. As shown in **figure 4.1**, the presence of HA- pp71 in the elute and not with the negative control confirms the interaction and no non-specific interactions.

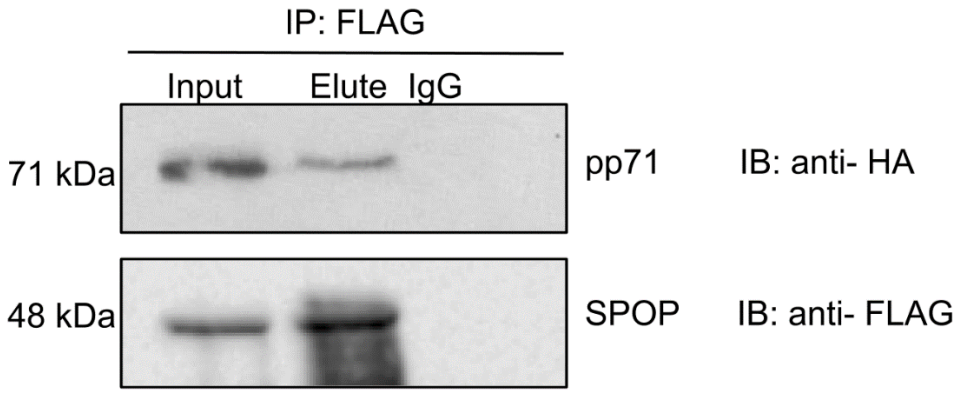



**Figure 4.1** pp71 interacts with SPOP. Co-immunoprecipitation performed in 1mg of U2OS whole cell lysates transfected with FLAG- SPOP and HA- pp71. The proteins are immunoprecipitated with protein A/G beads with anti- FLAG antibody and blotted with anti- HA for pp71 and anti- FLAG for SPOP. Input sample contains 10% of the amount of proteins used in experiments. *IP*, immunoprecipitate; *IB,* Immunoblotting

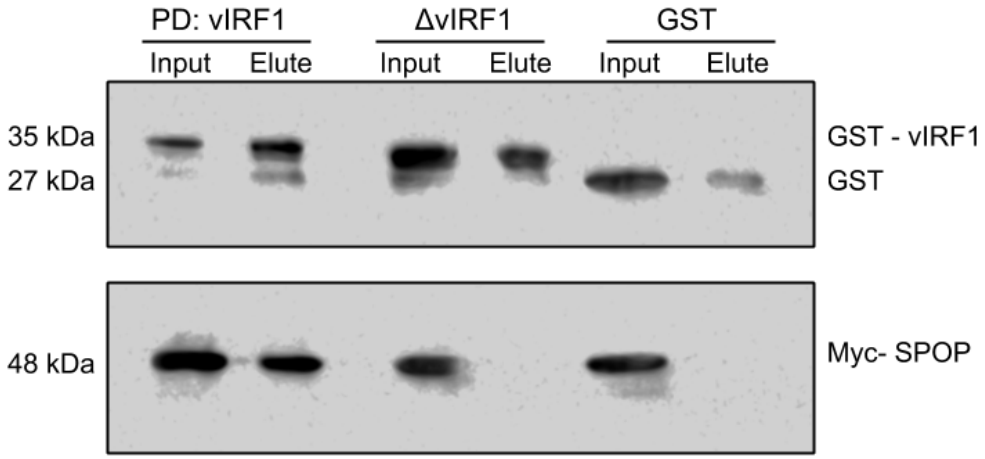
### 4.2.2 SPOP interacts with vIRF1 and vIRF3

The next family of proteins tested is vIRF proteins. Binary interaction between vIRF1 and SPOP was tested through a series of GST- pull down assays. Wildtype and mutant GST- vIRF1 peptides (residues 1 to 90) are expressed in bacteria and purified using glutathione beads as described previously. This sequence of vIRF1 was selected because it harbors the known USP7 binding site EGPS, if interaction indeed happen within the peptide, the binding site can be narrowed down to residues 1- 90. A GST- pull down experiment was performed using purified GST- vIRF1 bound to glutathione beads as bait and whole cell lysates of U2OS cells transfected with Myc- SPOP as prey. As shown in **figure 4.2 (a)** immunoblotting with anti- Myc identified Myc- SPOP in the elute, which indicated pull down of SPOP. We continued to examine whether the (PG)EGPS vIRF1 deletion mutant (ΔvIRF1) could interact with SPOP. The GST - pull down performed using purified ΔvIRF1 bound to glutathione beads as bait and using Myc-SPOP expressing cell lysates as prey. While the SPOP is present in the elute fraction with wildtype vIRF1, it is absent in the ΔvIRF1 elute fraction. This indicates that the deletion of the (PG)EGPS sequence abolished the interaction. A negative control was performed using GST protein alone; results indicate no binding between GST alone and SPOP, confirming vIRF1- SPOP interaction. Overall, these results confirm that vIRF1 interacts with SPOP *in vitro,* but ΔvIRF1 without the (PG)EGPS sequence does not.

This experiment was also conducted to show interactions *in vivo.* A Co- IP experiments was performed using whole cell lysates of U2OS cells co- transfected with Myc- SPOP with FLAG- vIRF1 or FLAG- ΔvIRF1. vIRF1 was Immunoprecipitation with anti- FLAG or mouse IgG antibody incubated with protein A/G beads. Co-

immunoprecipitation of SPOP was detected with immunoblotting using anti- Myc antibody. As shown in **figure 4.2 (b)**, SPOP was present in the elute fraction with vIRF1 but not in ΔvIRF1 elute. This suggests SPOP interacts with wildtype vIRF1 but not ΔvIRF1. To eliminate the possibility of non-specific interaction, the same experiment was conducted using mouse anti-IgG antibody. The results from this experiment showed that none of the tested proteins were in the eluted fraction. This confirmed that these proteins did not interact non-specifically with the beads. Interactions with USP7 was tested as a positive control, immunoblotting detected USP7 in the elute fraction with vIRF1 and not with ΔvIRF1.

(a)


PD: vIRF1
ΔvIRF1
GST
Input
Elute
Input
Elute
Input
Elute
35 kDa
27 kDa
GST - vIRF1
GST
48 kDa
Myc- SPOP

(b)

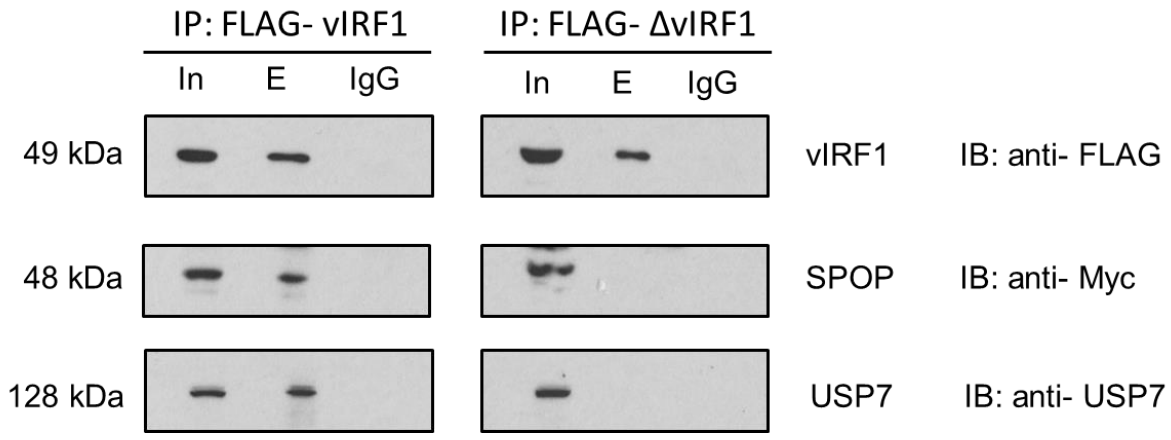

IP: FLAG- vIRF1
IP: FLAG- ΔvIRF1
In
E
IgG
In
E
IgG
49 kDa
vIRF1
IB: anti- FLAG
48 kDa
SPOP
IB: anti- Myc
128 kDa
USP7
IB: anti- USP7

**Figure 4.2 (a)** SPOP interacts with Wildtype vIRF1 but not ΔvIRF1 *in vitro*. GST- pull down comparing wildtype vIRF1 and mutant vIRF1: wildtype GST- vIRF (1-90) and GST-ΔvIRF1 (deleted PGEGPS) were purified and bound to glutathione beads as bait, Myc-SPOP from 500µg of whole cell lysates of U2OS cells were incubated with the beads as prey. Input sample contains 10% of the amount of proteins used in experiments. Samples are eluted and resolved on a 4-20% gradient SDS-PAGE and immunoblotted with anti-Myc and anti- GST. Negative control was performed using GST protein alone bound to glutathione beads incubated with cell lysates overexpressed with Myc- SPOP. **(b)** SPOP interacts with Wildtype vIRF1 but not ΔvIRF1. Co- IP experiment comparing wildtype vIRF1 and mutant vIRF1: U2OS cells were co- transfected with Myc- SPOP and FLAG-vIRF (1-90) or FLAG- ΔvIRF1 (deleted PGEGPS). 1 mg of whole cell lysates were used and vIRF1 was immunoprecipitated using anti- FLAG antibody and bound to protein A/G beads. Input sample contains 10% of the amount of proteins used in experiments. Samples are resolved on a 4-20% gradient SDS-PAGE. SPOP co-immunoprecipitation was detected through immunoblotting with anti- Myc antibody. Negative control was performed using anti- IgG antibody for immunoprecipitation. USP7 interaction was tested as a positive control.

Further, we examined other members of the vIRF family. As shown in the sequence alignment in **table 2.1**, vIRF3 also contains of the hypothesized binding motif EGPS. Interaction tests were done by using His- pull down. His- vIRF3 was purified and bound to nickel beads as bait. Whole cell lysate of U2OS cells transfected with Myc-SPOP was incubated with the His- vIRF3 beads as prey. SPOP is then identified through immunoblotting with anti- Myc. A positive control was performed by testing the interaction of a previously identified interactor of vIRF3, USP7. Endogenous levels of USP7 interacting with vIRF3 was detected using immunoblotting with anti- USP7 antibody in the elution fraction. The presence of USP7 in the elute confirms their interaction. A negative control was performed using empty nickel beads incubated with U2OS whole cell lysates expressing Myc- SPOP. Results are shown in **figure 4.3,** the presence of SPOP in the elute is indicative of the interaction between vIRF3 and SPOP. In addition, USP7 is also present in the elute confirming their interaction, while the absence of SPOP eluted from the empty beads indicates SPOP did not interact non-specially with the nickel beads.

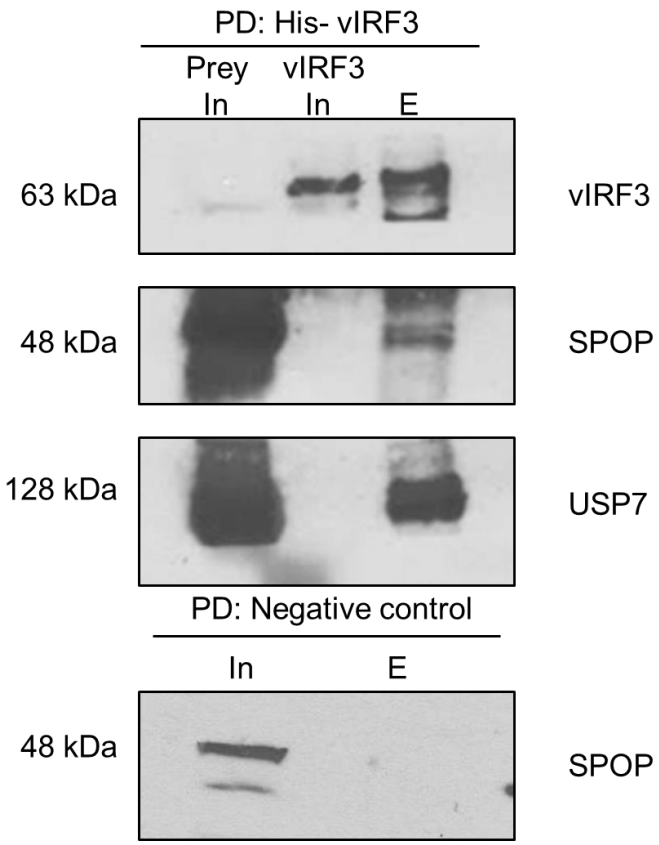



**Figure 4.3** vIRF3 interacts with SPOP. His - pull down performed using purified His-vIRF3 bound to nickel- beads as bait, incubated with Myc- SPOP whole cell lysate of U2OS cells as prey. Samples were eluted and resolved on a 4-20% gradient SDS-PAGE and immunoblotted with anti-Myc and anti- His antibodies. A positive control was performed by detecting endogenous USP7 through immunoblotting using anti- USP7 antibody. A negative control was performed using empty nickel beads incubated with U2OS whole cell lysates transfected with Myc- SPOP. Input sample contains 10% of the amount of proteins used in experiments. *In*, input; *E*, elute

### 4.2.3 SPOP Interact with EBNA1 and EBNA2

The next family of viral proteins tested is the EBNA proteins from Epstein- Barr virus. Results from sequence alignment analysis showed the conserved EGPS motif which is highly suggestive of interaction. A GST- pull down was performed using GST-EBNA1 peptide, residues 436 to 450. GST- EBNA1 was purified and bound to glutathione beads as prey. Purified His- SPOP- MATH protein was then incubated with the beads as prey. SPOP was identified using immunoblotting with anti- His antibody. As shown in **figure 4.4**, SPOP is present in the elute which indicated interaction. This also confirms that the interaction site can be isolate within residues 436 to 450 of EBNA1. A negative control was conducted using empty GST beads incubated with purified His- SPOP MATH protein, SPOP was not present in the elute fraction, indicating no non- specific interaction with the beads.

We next examined the interaction between SPOP and EBNA2. A co-IP experiment was performed using whole cell lysates of U2OS cells co- transfected with Myc-SPOP and FLAG- EBNA2. Whole cell lysates are then immunoprecipitated with anti- FLAG antibody and a negative control was performed using anti- IgG. The presence of SPOP is detected using immunoblotting with anti- Myc antibody. Results are shown in **figure 4.5,** which SPOP is detected in the elute, confirming their interaction. The lack of SPOP being immunoprecipitated with IgG indicates no unspecific binding.

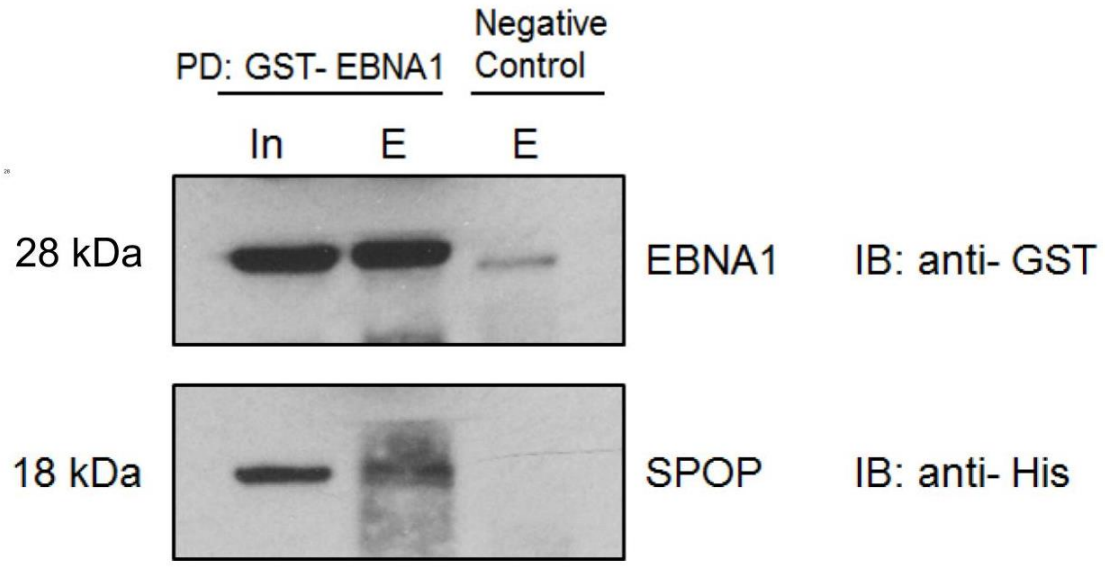



**Figure 4.4** EBNA1 interacts with SPOP. GST - pull down was performed using purified GST- EBNA1 (436 - 450) bound to glutathione beads as bait, incubated with purified His- SPOP- MATH protein. Negative control performed using empty glutathione beads incubated with purified His- SPOP- MATH protein. Samples are eluted and resolved on a 4- 20% gradient SDS- PAGE and proteins were detected through immunoblotting with anti- His and anti- GST antibodies.

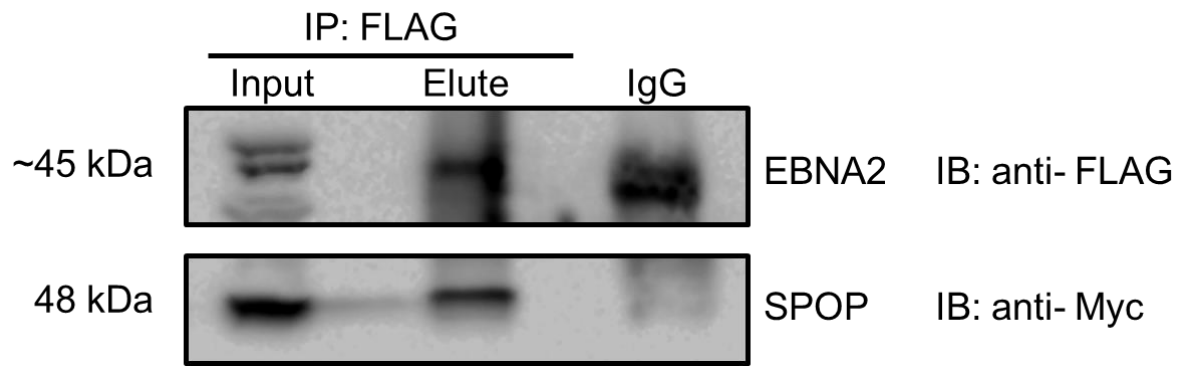
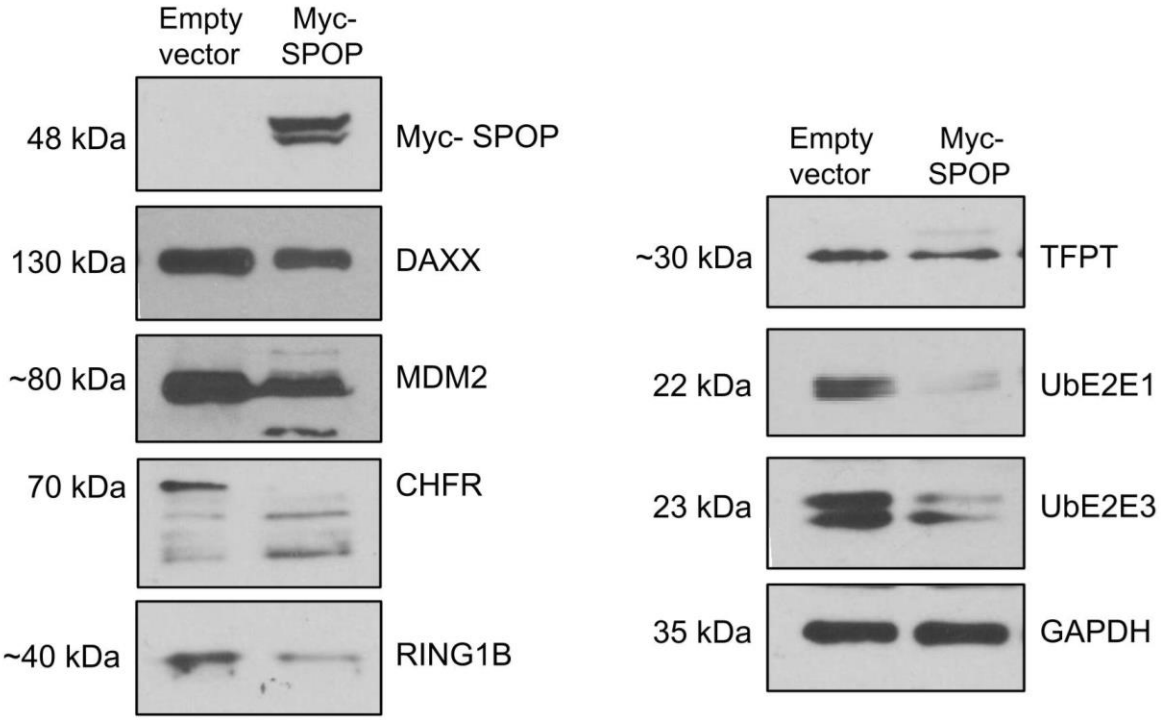



**Figure 4.5** EBNA2 interacts with SPOP. Co-IP performed using 1mg of whole cell lysates co- transfected with 5 ug of full-length FLAG- EBNA 2 and 5 ug of Myc- SPOP. Samples were immunoprecipitated using protein A/G beads with anti- FLAG antibody or anti- IgG antibody. Input sample contains 10% of the amount of proteins used in experiments. Samples were resolved on a 4- 20% gradient SDS PAGE and proteins were detected through immunoblotting with anti- FLAG and anti- Myc antibodies.

## 4.3 SPOP overexpression causes substrate destabilization

Upon confirmation of interaction between SPOP and above-mentioned protein, it is important to confirm whether these interacting proteins are substrates of CRL3- SPOP and if they are regulated by SPOP. SPOP, as part of an E3 ligase complex, functions to ubiquitinate its substrates, which will result in their degradation. Therefore, comparing cellular substrate levels with normal and overexpressed SPOP can reveal the effects of SPOP on its substrate. A western blot was performed comparing the cellular levels of identified cellular substrates Daxx, CHFR, MDM2, RING1B TFPT, UbE2E1 and UbE2E3 under normal levels and overexpressed SPOP. As shown in **figure 4.6 (a)**, upon increased SPOP level, there is noticeable decrease in the level of Daxx, CHFR, MDM2 and RING1B, TFPT, UbE2E1 and UbE2E3. Band intensities are then quantified using gel intensity analysis of ImageJ, averaged and normalized values are plotted in **figure 4.6 (b)**. Daxx was a previously identified SPOP substrate, CRL3- SPOP is known ubiquitinate to Daxx and decrease its stability (Kwon et. al, 2006). The results from this western blot is consistent with previous findings, we observed around 16% decrease in Daxx level of upon SPOP overexpression. MDM2 showed a 33.6% decrease, RING1B showed a 36.7% decrease, CHFR showed a 41.5% decrease, TFPT showed a 30% decrease and UbE2E1 and UbE2E3 both showed around 35% decrease. GAPDH levels were tested as a loading control, gel intensity analysis revealed no decrease in GAPDH levels upon SPOP overexpression, suggesting specificity of SPOP effects on its substrates.

**(a)**


Empty vector
Myc-SPOP
48 kDa
Myc- SPOP
130 kDa
DAXX
~80 kDa
MDM2
70 kDa
CHFR
~40 kDa
RING1B
Empty vector
Myc-SPOP
~30 kDa
TFPT
22 kDa
UbE2E1
23 kDa
UbE2E3
35 kDa
GAPDH

**(b)**

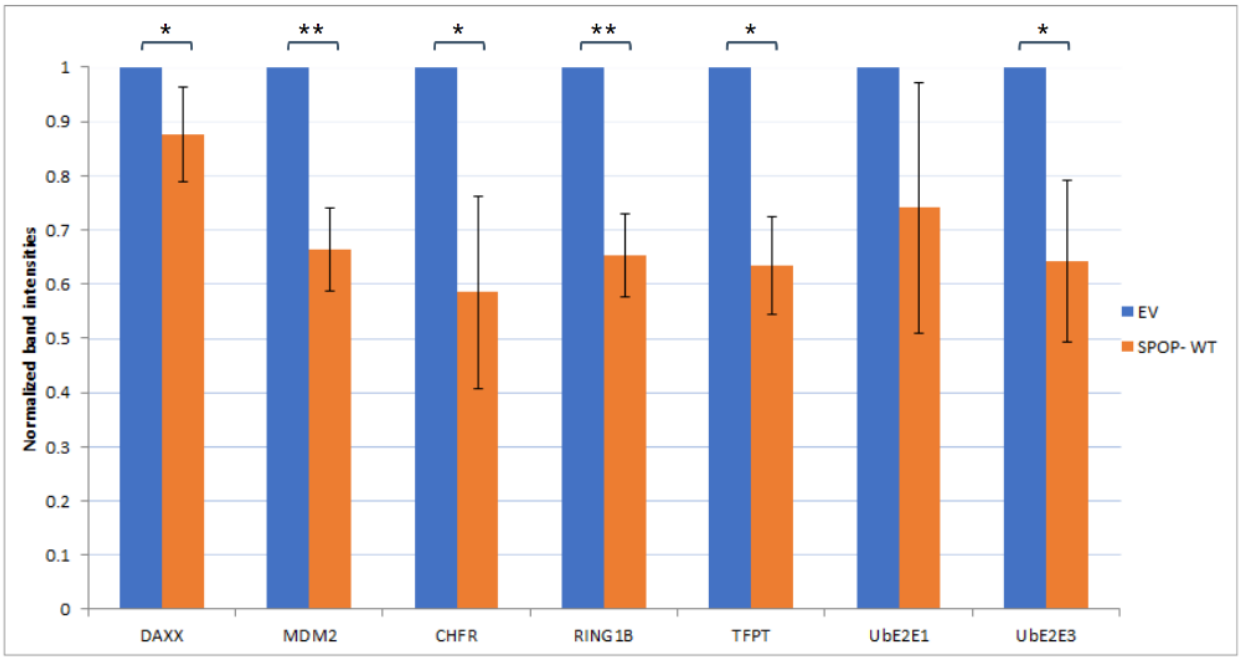

Normalized band intensities
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
*
**
*
*
DAXX
MDM2
CHFR
RING1B
TFPT
UbE2E1
UbE2E3
EV
SPOP- WT

**Figure 4.6 (a)** SPOP substrate levels under normal and overexpression of SPOP. Western blots showing the effect of SPOP overexpression on DAXX, MDM2, CHFR, RING1B, TFPT, UbE2E1 and UbE2E3 levels. The first lane is whole cell lysates of U2OS cells transfected with 5 ug of empty vector as control, and second lane transfected with 5 ug of Myc- SPOP. All cells are harvest 24 hours post transfection. **(b)** Histogram showing the average and normalized band intensity of each protein in **figure 4.6 (a)** under wildtype SPOP overexpression (SPOP- WT, orange) and control (empty vector, *EV,* blue) Individual band intensity are generated using the gel analyzing tool in ImageJ. All band intensities are normalized to their corresponding GAPDH (control) intensity. Band intensity of each protein under SPOP expression is then normalized to proteins under control levels. Error bars showing standard errors (n= 4- 11). A student t- test was conducted to determine significant difference, the following conventions were used: * ($p \leq 0.05$), ** ($p \leq 0.01$).

## 4.4 PROSTATE CANCER MUTATION AFFECTS SPOP SUBSTRATE BINDING

To test if prostate cancer mutation of SPOP is causing loss of substrate binding properties, a mutant SPOP was generated with the most common prostate cancer mutation, F133V, and tested for its ability to interact with its binding partners. The F133V mutation was introduced to the His- SPOP- MATH construct. A His- pull down experiment was performed with His- SPOP- MATH mutant as bait, under identical conditions as the previous His- pull downs. His- SPOP- MATH mutant protein was purified and bound to nickel beads incubated with whole cell lysates of U2OS cells as prey. A subset of previously identified proteins was selected and detected through immunoblotting. A His- pull down with wildtype His- SPOP- MATH was performed as comparison. As shown in **figure 4.7**, the selected substrates DAXX, RING1B and TFPT are detected in the wildtype SPOP elute but not in the mutant. This indicates that while the above-mentioned proteins are able to interact with wildtype SPOP, they cannot interact with mutant SPOP. In addition, a negative control was performed using empty nickel beads incubated with cell lysates, the absence of any proteins eluted indicates no nonspecific interaction with any protein.

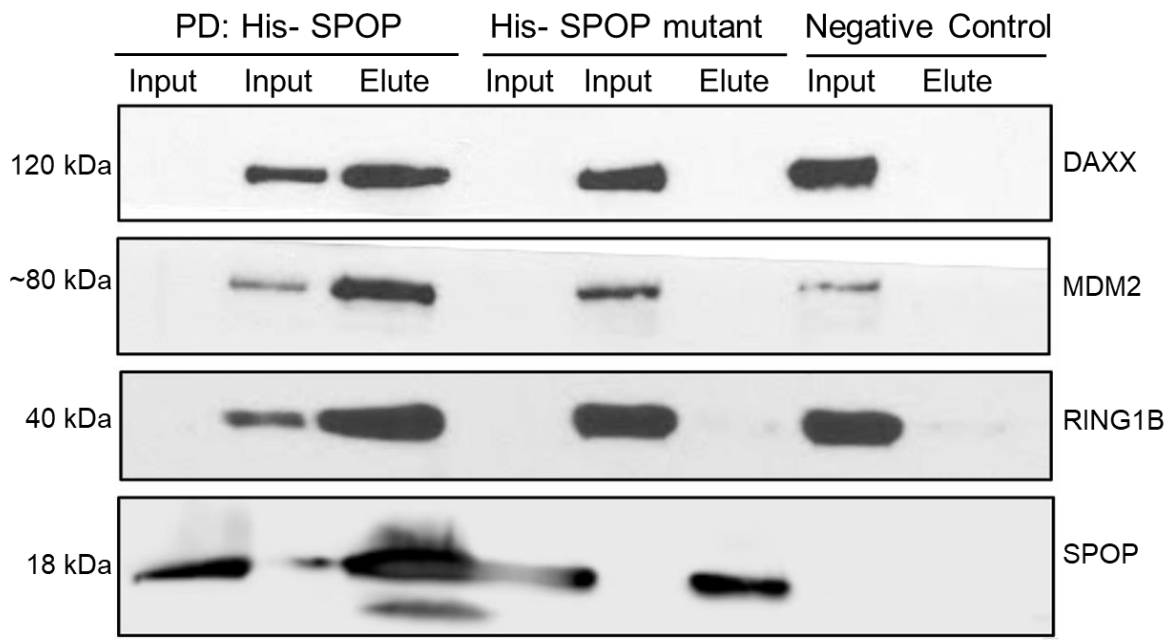



**Figure 4.7** His- pull down with wildtype and mutant SPOP (F133V). His- SPOP- MATH and mutant SPOP- MATH protein were purified and bound to nickel beads as bait. 500 ug of U2OS whole cell lysates were incubated with the resin as prey. A negative control was performed using U2OS cell lysates incubated with empty nickel beads. Samples are resolved on a 4-20% gradient SDS- PAGE and blotted with anti- His, anti- DAXX, anti- MDM2 and anti- RING1B antibody.

## 5. DISCUSSION

The main objective of this research is to identify potential substrates of CRL3-SPOP and if prostate cancer SPOP mutations affect their interactions. First, we aim to establish protein binding between SPOP and potential substrates. Results from various pull down and co- immunoprecipitation experiments identified six new cellular proteins that interact with SPOP: MDM2, CHFR, TFPT, RING1B, UbE2E1 and UbE2E3. We also identified five viral binding partners of SPOP: vIRF1, vIRF3, EBNA1, EBNA2 and pp71. After confirmation of interactions, our next aim was to examine how SPOP levels affect cellular protein levels. Results revealed that upon overexpression of SPOP, all above mentioned cellular proteins showed a decrease in protein levels. Lastly, we examined the effect that SPOP mutations have on these proteins. We found that mutant SPOP is no longer able to interact with previously identified binding partners.

Overall, this research identified potential substrates of CRL3- SPOP, identified new viral binding partners of SPOP and revealed that prostate cancer mutation of SPOP abolishes protein interactions. The cellular proteins interacting with SPOP reveal new roles of SPOP in different cellular pathways including cell cycle regulation, transcriptional regulation and cancer initiation. The viral binding partners indicate potential roles of SPOP in viral infection strategies.

## 5.1 CELLULAR SUBSTRATES OF SPOP

### 5.1.1 MDM2, RING1B, CHFR and TFPT are novel substrates of CRL3- SPOP

Through a series of biochemical and *in vivo* studies, we identified MDM2, RING1B, CHFR and TFPT to be SPOP binding proteins. Co- IP experiments confirmed their interactions *in vivo*, and His- pull downs using His- SPOP- MATH results indicate that the MATH domain alone is capable of facilitating the interaction. Furthermore, SPOP overexpression leads to decreased protein levels, which suggests that SPOP plays a role in their degradation. Given that SPOP is a Cul3 E3 ligase, MDM2, RING1B, CHFR, and TFPT are potential substrates of CRL3- SPOP which mediates their degradation through ubiquitination.

### 5.1.2 UbE2E1 and UbE2E3 interacts with SPOP but not UbE2E2

Similar results were observed in UbE2E1 and UbE2E3. Co- IP experiments were able to confirm binding between UbE2E1 and UbE2E3 with SPOP. In addition, the binding site of UbE2E3 resides on the N- terminal tail as it was able to interact with SPOP-MATH. Furthermore, both UbE2E1 and UbE2E3 showed decrease in protein levels upon overexpression of SPOP. This also suggests that they are substrates of CRL3- SPOP, which mediates their degradation. In comparison, UbE2E2 does not show interaction with full length SPOP nor isolated SPOP- MATH. Additional experiments with the N- terminal tail *in vitro*, also confirms lack of interaction.

## 5.2 VIRAL BINDING PARTNERS OF SPOP

A series of pull down and Co- IP experiments also identified several viral proteins that interact with full length SPOP, and with SPOP- MATH domain. vIRF1, vIRF3 and EBNA1 all showed interaction with the SPOP- MATH domain *in vitro*. The interaction between vIRF3 and SPOP was tested between full length vIRF3 and SPOP-MATH, through which we can infer that the SPOP MATH domain alone is able to facilitate the interaction. Binding was also observed between EBNA1 and SPOP- MATH. The binding site could be further narrowed down to the residues 436 to 450 in EBNA1. This is consistent with the previous interaction seen between EBNA1 and USP7, the USP7 binding site EGPS is located within this domain. The two viral proteins that were shown to interact with SPOP *in vivo*, are pp71 and EBNA2. Co- IP experiments performed using whole cell lysates expressing pp71 and EBNA2 showed interactions of the two proteins with SPOP.

The specific binding site of SPOP and its interacting proteins is demonstrated in vIRF1, which we identified to be a novel binding partner of SPOP. Interaction between vIRF1 and SPOP *in vivo* using full length vIRF1 as well as full length SPOP, and with N-terminal of vIRF1 and the SPOP - MATH domain alone was shown *in vitro* in the pull down experiments. More importantly, the binding site could be identified to be $^{47}$EGPS$^{50}$ through the mutational studies. In the pull down experiment, we compared a wildtype vIRF1 construct containing residues 1- 90 of the N- terminal DNA binding domain which harbors the hypothesized binding site $^{47}$EGPS$^{50}$, to the ΔvIRF1 construct, residues 1- 90 with a deletion mutation at $^{45}$PGEGPS$^{50}$ residues. Our results showed that wildtype vIRF1 is able to interact with SPOP but ΔvIRF1 without the PGEGPS sequence does not interact

with SPOP. This confirms that the vIRF1 (PG)EGPS sequence is the crucial binding site that interacts with the SPOP- MATH domain.

## 5.3 SPOP BINDING MOTIF

The sequences of the newly identified binding partners of SPOP are mostly consistent with the previously established SPOP binding motif (φ-π-S-S/T-S/T) which interacts with the SPOP- MATH domain. However, the constriction on the third residue being only S is debatable. MDM2 harbors the PSTSS sequence, which mostly matches the binding motif, except for the T instead of S at the third residue. Similarly, CHFR also contains a sequence which mostly matches the motif, PSTST, which also has a T at the third residue. This is also the case for UbE2E3, which also has a T at the third residue and BMI1, a previously identified SPOP substrate, also contains the binding site PSTSS with a T at the third residue. This suggests that the third residue of the motif could be either S or T. In the case of RING1B, the predicted SPOP site and known USP7 binding site is ATASG, which differs from the SPOP binding motif except for T and S. It seems that the USP7 motif E/P/AxxS is also a possible SPOP binding motif. This suggests that it is the last three or four residues that are important in order to facilitate binding.

As mentioned previously, UbE2E2 is not a binding partner of SPOP while UbE2E1 and UbE2E3 are. Looking at the binding site sequence of UbE2E2 compared to UbE2E1 and UbE2E3, the only difference is in the fifth residue. Instead of the S or T in UbE2E1 and UbE2E3, a G was the fifth residue. Looking at the structure of the amino acids, S and T are polar, uncharged residues with a hydroxyl group to facilitate interaction, while G is a non- polar residue and is unable to facilitate binding. This suggests that one residue is

able to abolish the interaction with SPOP, and also indicates that the fifth residue plays an important role in facilitating binding.

This difference in one residue seems to distinguish the binding motif between SPOP and USP7. Looking at the previously identified USP7 binding motif, proteins containing EGPS also show an interaction with SPOP. All of the viral proteins tested and TFPT harbor the EGPS motif, which suggests that EGPS is a binding motif for SPOP as well. As demonstrated in the vIRF1 pull down experiment, the EGPS motif is crucial to facilitate an interaction with SPOP. Looking at the sequence of the identified binding partners of SPOP, only the EGPS motif is conserved, no major pattern could be observed in the previous or the following residues. Unlike the previously identified φ-π-S-S/T-S/T motif, the fifth residue does not seem to play an important role in binding. Therefore, EGPS can also be established as an SPOP binding motif.

The large number of shared substrates between SPOP and USP7 is intriguing as the two proteins have opposing functions. SPOP, as part of an E3 ligase, is responsible for promoting protein degradation, while USP7 is a deubiquitinating enzyme that rescues proteins from degradation. The only USP7 substrate that did not show interaction with SPOP is UbE2E2, likely due to the difference in one residue. This built in distinction between the two binding motifs suggests that there are differences in how USP7 and SPOP select and recruit their substrate.

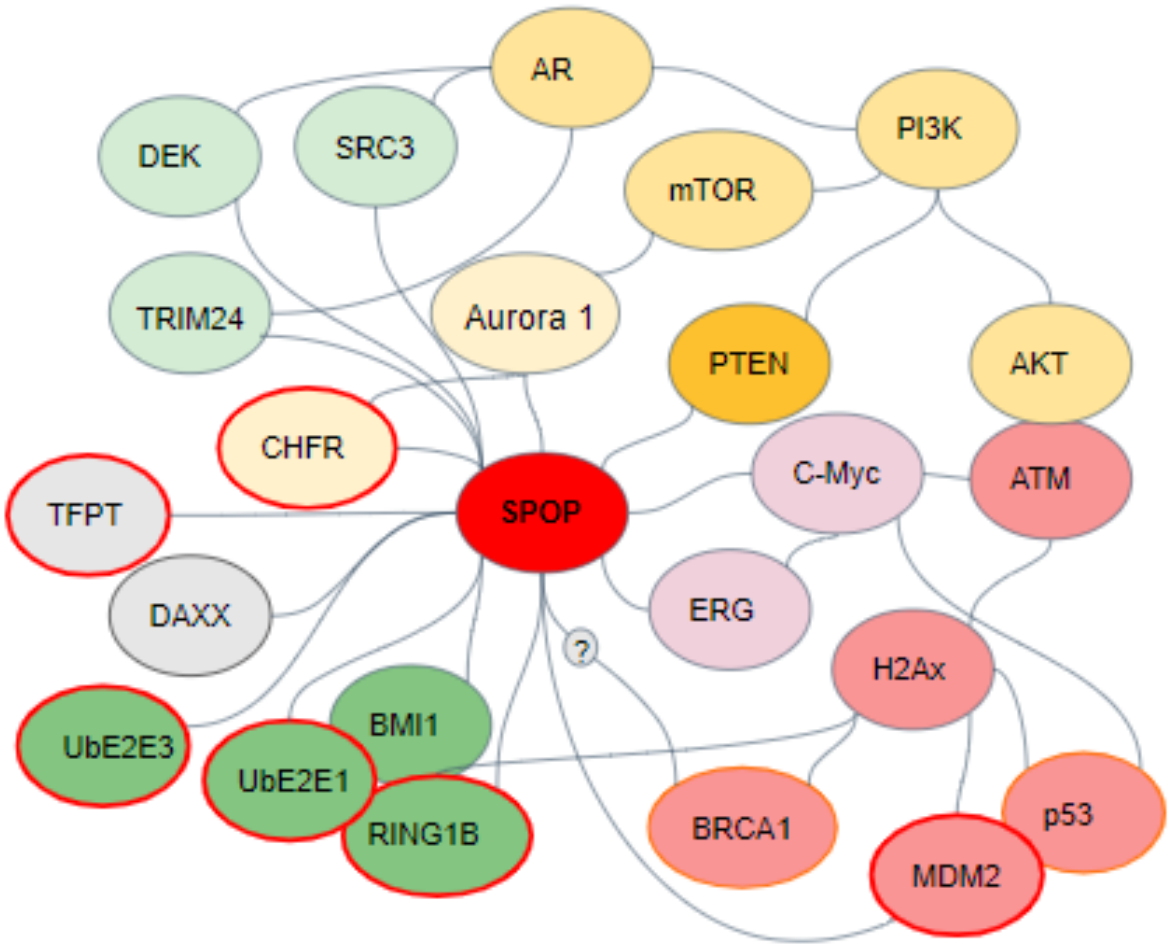



**Figure 5.1** Partial SPOP interacting map and pathways. This demonstrates the previously identified and new identified substrates (in red circle) or binding partners of SPOP. It is previously known that SPOP is implicated in the PI3K/mTOR pathways (yellow) dysregulation which leads to prostate cancer. Important SPOP substrates previously identified includes PTEN, ERG, c- Myc and Aurora1, and proteins known to regulate AR activities including SRC3, DEK and TRIM24 (blue). This study identifies new substrate of SPOP in the PRC1, including RING1B, UbE2E1 and previously known BMI1 (green). This complex is also known to overlap with the DNA damage and repair pathways (pink) through monoubiquitination of H2AX. MDM2 is also a part of this pathway and a newly identified SPOP substrate. Other proteins include CHFR and TFPT.

## 5.4 POTENTIAL TUMORIGENIC MECHANISM OF SPOP MUTANT

Among all cellular substrates identified, there are a few known proteins associated with cancer. Experiments were carried out to test the effects of prostate cancer SPOP mutation *in vitro* in terms of protein- protein interactions.

### 5.4.1 SPOP Mutant Abolish Protein Interactions

The most common prostate cancer mutation of SPOP is F133V. The effect of this mutation was examined in terms of its binding. Various pull down experiments demonstrated that the SPOP MATH domain with the sequence DWGV no longer interacts with the previously identified substrates. This suggests that the F residue in the substrate binding site (DWGF) is critically important for establishing an interaction. This is consistent with previous findings in which prostate cancer SPOP mutations occurring in the substrate binding site leads to loss of SPOP- substrate interaction. This could affect various cellular pathways as discussed below.

### 5.4.2 MDM2 interacts with SPOP but not with mutant SPOP

This study identifies MDM2 as a binding partner of SPOP and a potential substrate of CRL3- SPOP. We demonstrated that MDM2 protein levels decrease upon SPOP overexpression and SPOP mutant does not interaction with MDM2 and therefore unable to regulate its levels. MDM2 is a potent oncogene which is a key negative regulator of p53. p53, as a tumor suppressor, plays an essential role in preventing tumor formation. This suggests that prostate cancer SPOP mutation could leads to increasing stability of MDM2, which results in its accumulation. In turn, this accumulation of MDM2 can cause increased degradation of p53, which leads to cancer initiation. In various tumors,

increased levels of MDM2 have been seen, which includes both an increase in transcriptional levels (Momand et. al, 1998) and post- translational modifications (Khoronenkova et.al, 2012). It is, therefore, possible that in prostate cancer, tumorigenesis is caused by SPOP mutation which leads to increased MDM2 levels, which may in turn decrease p53 levels.

Since we demonstrated that levels of MDM2 could be regulated by SPOP, dysregulation due to SPOP mutation is a potential oncogenic pathway. Increased cellular levels of MDM2 could cause p53 degradation, leading to cancer initiation. To date, the majority of the treatments surrounding MDM2 and p53 involve small- molecule inhibition of the interaction between p53 and MDM2 in order to reduce degradation of p53 by MDM2 (Vassilev et. al, 2004). This inhibition could potentially benefit the case of MDM2 accumulation. Alternatively, targeting SPOP mutation would effectively maintain MDM2 at its optimum level.

### 5.4.3 CHFR Interacts with SPOP but not with Mutant SPOP

CHFR is also identified as a novel substrate of CRL3- SPOP. It interacts with SPOP and is downregulated by SPOP. CHFR is also an identified cancer biomarker for its function in regulating cell cycle progression by controlling their mitotic entry (Scolnick et. al, 2000). Previous research identified hypermethylation in the promoter of CHFR or downregulation of CHFR to be associated with cancer, as the downregulation of CHFR leads to chromosomal instability and causes tumor formation (Hanahan et. al, 2011). It is known that USP7 stabilizes CHFR levels through deubiquitination which rescues it from degradation (Oh et. al, 2007), and that upregulation of CHFR is associated with tumor

suppression (Zhang et. al, 2017). Having discovered that CHFR is an CRL3- SPOP substrate, SPOP is functioning against USP7 to decrease CHFR levels.

In cases like pancreatic cancer, the accumulation of CHFR was shown to inhibit cancer progression (Zhang et al. 2017). It is also known that SPOP overexpression in the cytoplasm becomes a regulatory hub to promote tumorigenesis (Li et al. 2014). In this case, SPOP is also a potential tumor promoter through ubiquitination and degradation of cellular proteins such as CHFR as well as PTEN. There has been success in developing a small molecule inhibitor to block the interaction between SPOP and PTEN (Guo et. al, 2016). CHFR as a novel binding partner of SPOP also has a similar binding motif to PTEN. This suggests that the small molecule inhibitor discovered by Guo et. al for PTEN binding could be effective on inhibiting the CHFR and SPOP interaction as well. This suggests that the drug's effectiveness in kidney cancer treatment could also be due to CHFR inhibition in addition to PTEN inhibition.

### 5.4.4 SPOP and PRC1

Novel interacting partners of SPOP include histone- associated proteins. In particular, there are many substrates involved in PRC1 which regulates the ubiquitination of histone H2A in epigenetic and transcriptional regulation. RING1B is a PRC1 subunit, as an important E3 ligase it is known that the activation and regulation of RING1B both rely on ubiquitination. Self- ubiquitination or ubiquitination via exogenous E3 ligase both activate RING1B. USP7 was believed to keep RING1B in its "native state" through constant deubiquitination (Bie et. al, 2010). This allows the PRCs or the upstream

determinants of RING1B to regulate its ubiquitination level, which in turn determines its activities.

It was previously believed that the state of ubiquitination of RING1B might be due to an unknown E3 ligase. Based on the results of this research, SPOP interacts with and downregulates of RING1B, suggesting that SPOP could potentially be the upstream E3 ligase mediating RING1B ubiquitination as well as degradation. In addition, it was previously demonstrated that SPOP regulates BMI1 levels, which is also an important regulator of RING1B. Altogether, SPOP could be the critical upstream regulator in regulating the level of ubiquitination in RING1B. UbE2E1 was also identified as a subunit of PRC1 as a critical E2 enzyme, it is also a known interactor with RING1B and is known to be regulated by USP7. While the role of USP7 in PRC1 activity remains ambiguous, SPOP may be functioning against it, thus regulating ubiquitination levels and protein levels in order to regulate the activity of each component **(figure 5.2**).

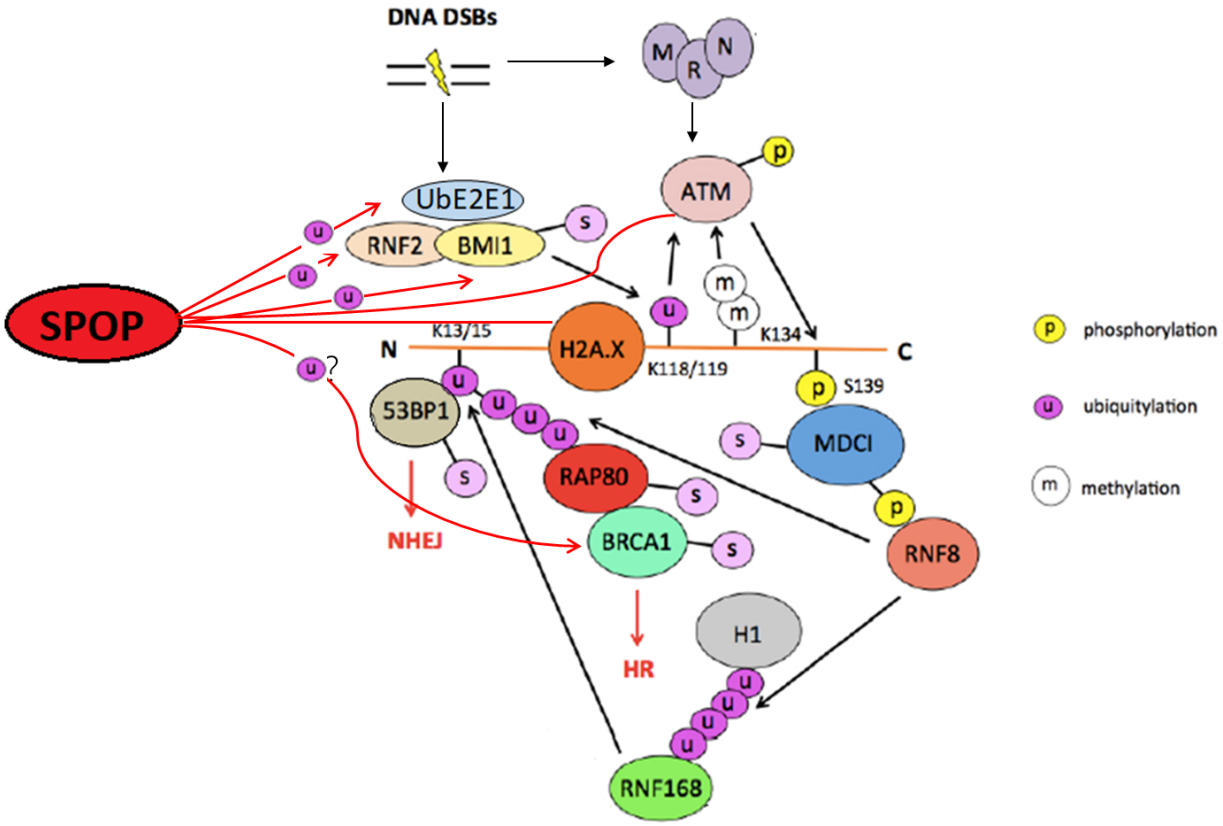



**Figure 5. 2** SPOP's association in multiple signaling pathways. SPOP is previously found to interact with BMI1 and currently shown to interact with the rest of the PCR1 complex RING1B (RNF2) and UbE2E1. Additionally, SPOP is known to interact or function along ATM and H2AX in DNA damage repair, BRCA1 is also a potential substrate of SPOP. (Adapted and modified from Jessica D' Angelo, unpublished 2018)

## 5.5 SPOP's interactions with viral proteins

It was previously unknown that SPOP could interact with any viral proteins. No experiments have been carried out on testing specific binding sites for the viral proteins, however based on the viral protein sequence as well as its similarity to USP7- viral protein binding, we can presume that SPOP can also interact with its viral binding partners at its DWGF motif and at the same binding sites as cellular substrates. This suggests that there could be competitive binding between the viral proteins and cellular substrates for SPOP. Leading to the possibility that cellular proteins are not efficiently regulated upon viral infection. It was previously discovered that vIRF proteins as USP7 binding partners have an inhibitory effect on p53 as it competes for USP7 binding on the same site. This in turn contributes to IFN invasion (Nakamura et. al, 2001; Chavoshi et. al, 2016).

The large variety of viral SPOP substrates also suggests an alternative route in cancer development. Previous research suggests that viruses such as EBV have oncogenic potential and that EBV, along with other virus such as human papillomavirus (HPV), could enhance cancer cell proliferation. Sequencing results of prostate cancer specimens revealed that EBV genes are presented similarly in normal, benign and prostate cancer tumors, which appears to be ubiquitous. However, there is a significantly higher presence of both EBV and HPV in tumor samples (55%) than in benign (15%) or normal (30%) samples (Whitaker, 2012). This suggests that HPV could exerts its oncogenic influences collaborating with EBV, potential functions of SPOP involved in viral protein regulations are yet to be determined.

## 5.6 FUTURE DIRECTIONS

It can be concluded that SPOP is involved in multiple pathways that could lead to cancer formation and progression. In the future, more experiments can be done on investigating the mechanism of the SPOP- substrate binding, prostate cancer SPOP mutant on histone modification through PRC1 complex, as well as on the DNA damage repair pathway, and viral infection.

The mechanism of SPOP binding could be better understood through NMR, which would confirm the interaction and reveal the residues involved in binding. The NMR spectrum of wildtype SPOP and mutant SPOP could be compared to identify differences in substrate binding on a residue level. In addition, multiple mutations to the SPOP binding motif could be tested. Individual residues of the DWGF motif could be mutated to see changes in binding properties, mutations besides F133V may also cause disruption in substrate binding and how the prostate cancer mutation can be rescued. In the case of USP7, a double mutant of $^{164}$DWGF$^{167}$ is changed into $^{164}$AAGF$^{167}$. This mutation eliminated the binding to substrates such as UbE2E1 (Sarkari, 2013) and vIRF1 (Chavoshi, 2016). Other versions of SPOP mutants could be tested, a few interesting ones would be AAGF, DGGF and DGGV.

It is also important to understand the binding affinities between SPOP and its substrates. Fluorescence polarization could be done in order to measure the dissociation constant between the different peptides. The competitive binding between the cellular substrates and viral substrates could be revealed upon knowing which substrates have a higher binding affinity. Another potential interesting comparison would be to see the

binding affinity between SPOP- substrates and USP7- substrates. Since USP7 and SPOP have opposing effects on its substrate, the nature of the interactions could reveal if a substrate is more likely to be ubiquitinated or deubiquitinated. Viral substrates, in particular, are known to have a higher affinity to bind USP7 compared to cellular substrates.

To further study the role of SPOP on the p53 pathway, the levels of p53 can be tested on mutant SPOP expression, vIRF expression, MDM2 overexpression, etc. Additional downstream p53 targets such as p21 and BAX can also be studied. To do so, p53 null cells can be used as well as transfected with SPOP vector, mutant SPOP vector, vIRF vector in different combinations with and without p53. These experiments can then reveal the ability of p53 to activate transcription. Further studies on p53 knockout mice could then be used to study tumorigenesis. To further investigate the role of SPOP, subcellular localization of SPOP, USP7, viral proteins and IFN pathway protein could be seen at different stages of viral infection, through the use of immunofluorescence and confocal microscopy. This could reveal the co- localization pattern of the proteins as well as reveal a spatial and temporal arrangement.

Additionally, there are many other potential SPOP substrates yet to be tested. Viral proteins such as vIRF2 as well as vIRF4 are also known USP7 interacting proteins and harbor the EGPS binding consensus. If SPOP interacts with more proteins in the KHSV family, it is possible that SPOP is involved in KHSV infection. Other binding partners include viral protein ORF45 as well as cellular proteins TERT and BRCA1. As mentioned previously, BRCA1 is associated with many DNA repair processes including NHEJ as well as HR and also integrates signals from different histone modifications. SPOP is a

potential regulator of BRCA as it was previously demonstrated that BRCA1 levels increase in breast cancer with SPOP mutations. BRCA1 is also a known USP7 substrate, which means it will likely interact with SPOP as well. This suggests SPOP could have a more prominent role in regulating DNA damage and repair, as well as integrating signals from histone modifications.

SPOP mutation could be tested *in vivo* using prostate cancer cells. Continuing from our current findings, it would be useful to test if SPOP mutation would cause changes in protein levels for the identified substrates. Differing from other mammalian cells, certain prostate cancer cells exhibits unique characteristics such as AR sensitive, harbor certain mutations in genes encoding for p53 or PTEN. This can be a better indication of prostate cancer SPOP mutation at its original source and understand the combined effects of occurring pathways. In comparison, the same experiment should be done in non- prostate cancer cells, to confirm effects of SPOP mutation independent of other AR signaling or other pathways.

Mouse models carrying the SPOP prostate cancer mutant have demonstrated its impact on tumorigenesis *in vivo*, leading to the observation that it is due to the loss of PTEN as well as the activation of PI3K/mTOR and AR signaling (Blattner et. al, 2017). In addition to the previously identified pathway, alterations in other pathways previously not known to be implicated in prostate cancer can be tested. Protein level changes in MDM2 and p53 pathways as well as downstream effectors of p53 could be tested. Changes in protein levels of members of the PRC1 complex and their targets can also be investigated, as well as DDR proteins. Protein levels combined with different phenotypical changes could reveal a more distinct molecular mechanism for cancerous phenotypes. It is

important to identify the distinct roles of the SPOP mutant in tumorigenesis and tumor progression. To test for tumorigenesis, SPOP mutant can be conditionally expressed and introduced to mouse prostate organoids, as described by Blattner et. al., in order to see the levels of the epithelial- mesenchymal transition markers simultaneously with the above- mentioned protein levels and phenotypic changes. In comparison, SPOP mutant can also contribute to generating an environment optimal for cancer cell proliferation and tumor metastasis. Mutant SPOP can be conditionally expressed in pre-existing tumors of immunodeficient mice, and observe how SPOP mutations lead to tumor cell growth, increases in invasiveness and metastasis, etc.

SPOP mutation is one of the most common point mutations clinically localized to prostate cancer. This study identifies new substrates that were previously unknown to interact with SPOP. All cellular substrates identified to interact with SPOP either directly or indirectly link to cancer initiation or progression. This greatly expands the previously identified pathways of tumorigenesis and tumor progression. We also identified new viral binding partners of SPOP which suggests that SPOP plays a role in regulating viral infection and virus- caused cancer. Taken together, identifying new substrates and binding partners for SPOP could allow us a better understanding of SPOP's function in protein regulation, which reveals how changes in protein levels upon SPOP dysfunction leads to tumorigenesis as well as tumor progression. Therefore, the identification of a variety of SPOP targets in different pathways, could lead to alternative routes for developing cancer treatments.

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