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Role of ubiquitination in IGPR-1 regulation

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ROLE OF UBIQUITINATION IN IGPR-1 REGULATION

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ROLE OF UBIQUITINATION IN IGPR-1 REGULATION

LINZI SUN

ABSRACT

Immunoglobulin-containing and proline-rich receptor-1 (IGPR-1), is a newly identified cell adhesion molecule and is expressed in various cell types including, epithelial and endothelial cell origin. IGPR-1 regulates cell-cell adhesion and promotes angiogenesis, activated by shear stress and mediates endothelial cells response to shear stress. Moreover, IGPR-1 expression is upregulated in colon cancer and supports colon tumor growth. IGPR-1 contains a single extracellular immunoglobulin domain, a transmembrane domain and followed by a cytoplasmic proline-rich C-terminus.

We demonstrate that ubiquitin E3 protein ligase neural precursor cell expressed developmentally down regulated 4 (NEDD4) binds to and ubiquitinates IGPR-1. Furthermore, among the four WW domains, the C-terminus WW domain#4 selectively mediates the binding of NEDD4 with IGPR-1. We used *in vitro* ubiquitination assay and identified UbcH6, as an E2 conjugating enzyme required for NEDD4-mediated ubiquitination of IGPR-1. Taken together, our data identifies NEDD4/Ubc6H ubiquitination system as a major pathway involved in the ubiquitination of IGPR-1.

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LIST OF ABBREVIATIONS

ACK ..... Cdc42-associated tyrosine kinase

AJs ..... adherence junctions

AMP ..... adenosine monophosphate

AMPAR ..... alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors

Arp2/3 complex ..... actin-related proteins Arp 2 and Arp 3

BBB ..... blood-brain barrier

BPAG1 ..... bullous pemphigoid antigen 1

CACNB2 ..... and calcium channel β2 subunit

Co-IP ..... co-immunoprecipitation

CP ..... core particle

E6-AP ..... E6- associated protein

EAE ..... experimental autoimmune encephalomyelitis

EB ..... bullous epidermolysis

EGFR ..... epidermal growth factor receptor

ENaC ..... amiloride-sensitive epithelial sodium channel

ErbB4 ..... type I transmembrane receptor tyrosine kinase

FGFR1 ..... fibroblast growth factor receptor 1

GAPDH ..... glyceraldehyde-3-phosphate dehydrogenase

GBM ..... glioblastoma multiforme

HECT ..... homology to the E6-associated protein carboxyl terminus

HIV ..... human immunodeficiency virus

HSAN-VI .....hereditary sensory autonomic neuropathy type VI

IGPR-1 ..... immunoglobulin and proline-rich receptor-1

JAMs ..... junction adhesion molecules

L-VDCC .....L-type Ca2+ -channels

mDia .....diaphanous-related formin-1

MIA ..... melanoma inhibitory activity

MUPP1 .....multi-PDZ domain protein

NEDD4 ..... neural precursor cell expressed developmentally down regulated 4

non-PY motif .....non-canonical sequence

PAR-3 .....partitioning defective protein 3

PAE .....porcine aortic cells

PATJ ..... pals1-associated tight junction protein

PEBP2 .....polyoma enhancer binding protein 2

PRDs .....proline-rich sequences

PTEN ..... phosphatase and tensin homolog

RING .....really interesting new gene

RP ..... regulation particle

Ser220 ..... serine 220

SH3 .....Src homology 3

SIRT2 ..... sirtuin 2

SPIN90/WISH ..... SH3 protein interacting with Nck

TEER ..... trans-endothelial electrical resistance

TJ ..... tight junction

VE-cadherin ..... vascular endothelial-cadherin

YAP ..... yes kinase-associated protein

ZO-1, -2, -3 ..... zonal closure proteins 1, 2, and 3

INTRODUCTION

To live, all vertebrate animals have evolved to have a circulatory system, and endothelial cells are the main components of blood vessels. In addition to its essential function of transporting oxygen, they also govern the immune response, hemostasis, angiogenesis, and vascular permeability (Rahimi, 2017). Endothelial cells are able to open a path for transmigration and guide leukocytes to extravascular tissues (Vestweber, 2015). Furthermore, endothelial cells are necessary to sustain the blood fluidity and prevent thrombus formation. They can control the hormones, solutes, extravasation of fluid, and macromolecules, as well as guarantee the appropriate amount of clotting factors and platelets in blood vessels (van Hinsbergh, 2012). In addition, the integrity of endothelial cells is indispensable to retain the highly dynamic barrier function of circulatory system. Its dysfunction is associated with significant human diseases and the outcome of the surgery. Considering the multiple functions of endothelial cells, a plethora of molecules, including claudin family proteins, junction adhesion molecules (JAMs), vascular endothelial (VE)-cadherin, and immunoglobulin and proline-rich receptor-1(IGPR-1) are involved in regulating their function and integrity (Rahimi, 2017).

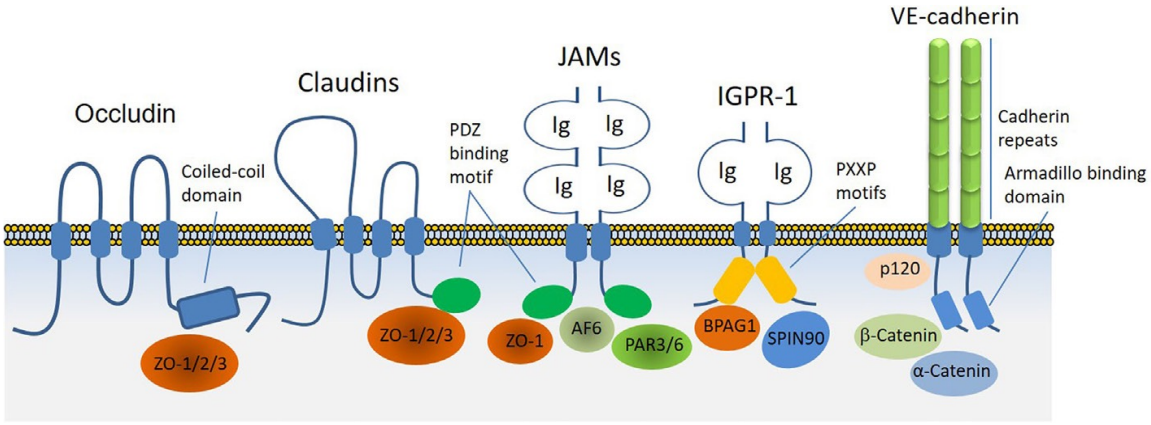


Figure1. **Transmembrane receptors involved in the endothelial cell–cell junction and their key cytoplasmic-binding partners (Rahimi, 2017).**

Claudins

Claudins are transmembrane proteins that represent the most critical components of the tight junction (TJ). Claudins contains four transmembrane domains in the N-terminal and a C-terminal cytoplasmic domain consisting of 255 amino acids (Furuse et al., 1994). Claudins can associate with the TJ network to form multiple proteins through its cytoplasmic domain. Zonal closure proteins 1, 2, and 3 (ZO-1, -2, -3), the multi-PDZ domain protein (MUPP1), and the Pals1-associated tight junction protein (PATJ) are all capable of interacting with the cytoplasmic C-terminus of claudins (Ruffer, 2004). Several functions of claudins in physiology and pathology have been reported. For example, the lack of the TJ barrier function caused by the absence of claudins has led to a series of diseases characterized by increased tissue permeability. Research demonstrates the loss of claudin-3 when the integrity of the blood-brain

barrier (BBB) is impaired under pathology conditions such as experimental autoimmune encephalomyelitis (EAE)or glioblastoma multiforme (GBM). Thus, explains the importance of claudin-3 as a vital component determining the integrity of BBB TJs in vivo (Wolburg et al., 2003). In addition, claudins expression in colonic mucosal epithelial cells from some Crohn's disease patients is significantly down-regulated (Kucharzik et al., 2001). Furthermore, mutations of claudins can cause multiple human diseases. For instance, claudin-16 mutation is closely related to familial hypomagnesemia with hypercalciuria and nephrocalcinosis, while claudin-14 mutations were documented to be associated with non-syndromic recessive deafness DFNB29 (Weber et al., 2001) (Wilcox et al., 2001). Interestingly, many studies have reported that claudins are also regulated by various factors during the tumor development and progression. Comparing to normal cells, Claudin-23 is down-regulated in breast, gastric, and pancreatic cancer cells, on the other hand, the expression of claudin-7 is down-regulated in head and neck squamous cell carcinoma (Moustafa et al., n.d.) (Kominsky et al., 2003).

Junction adhesion molecules (JAMs)

Junction adhesion molecules are a family of proteins on the cell surface that are involved in cell-cell adhesion and barrier function. JAMs contain a cytoplasmic domain, a transmembrane domain, and two extracellular immunoglobulin folds

(Bazzoni, 2003). JAMs are selectively expressed at the intercellular junctions of endothelial cells and epithelial cells, especially enriched at tight junctions (Martìn-Padura et al., 1998). Prior data have shown that a blocking monoclonal antibody against JAM can inhibit the transendothelial migration of monocytes, demonstrating that JAMs play a vital role in mediating the leukocytes and endothelial cells interaction (Del Maschio et al., 1999) (Bazzoni et al., 2000). Besides, JAM-A and JAM-C could stimulate platelet aggregation, as well as the interaction of platelets to the endothelium and leukocytes (Ebnet, 2004). Another essential role of JAMs is based on the fact that JAMs contain type II PDZ domain binding motifs --a protein-protein interaction module that plays a central role in organizing various cell signaling assemblies (Songyang, 1997) (Harris et al., n.d.). JAMs recruiting TJ components such as ZO-1， MUPP1 and cell polarity protein PAR-3 in a PDZ-domain-dependent manner strongly suggests that JAMs regulates TJ formation during the acquisition of cell polarity (Ebnet, 2004).

Vascular Endothelial cadherin

Vascular Endothelial cadherin（VE-cadherin）is one of the main families of cell adhesion proteins expressed in endothelial cells, which belongs to the type II cadherins. Same as other subtypes of cadherins, VE-cadherin has a highly conserved cytoplasmic tail, a transmembrane region, and five extracellular cadherin domains

(Vestweber, 2008). Cytoplasmic C-terminal of VE-cadherin is the binding site of p120-catenin, β-catenin, and plakoglobin (Giannotta et al., 2013). The association of VE-cadherin and the above proteins is necessary for the constitution of Adherence junctions (AJs). It is worth mentioning that the adhesive property of AJ is affected by VE growth factors, inflammatory mediators, angiopoietin 1, and Cyclic AMP by regulating the phosphorylation of VE-cadherin (Fukuhara et al., 2005). In early embryonic development, VE-cadherin is involved in the early assembly of vascular structures and early cardiovascular development (Breier et al., 1996). Except for the role of VE-cadherin mentioned above, VE-cadherin also contributes to the quiescent state of AJ during contact inhibition (Giannotta et al., 2013).

Immunoglobulin-containing and proline-rich receptor-1:

Immunoglobulin-containing and proline-rich receptor-1 (IGPR-1), is a newly identified cell adhesion molecule (Rahimi et al. 2012). Studies have found that IGPR-1 is restrictedly expressed in higher mammals, including primates, dolphins, and canines, but not in rodents, such as rats and mice. It seems to prove that this molecule evolved to perform a specific function in higher mammalians as it is different from other classical cell adhesion molecules, which are widely present in different species (Rahimi et al. 2012). In addition, IGPR-1 expression is predominantly found in endothelial cells and epithelial cells, especially in multiple human organs/tissues such

as colon, small intestine, skin, kidney, skeletal, heart muscle, and brain (Rahimi et al. 2012).

IGPR-1 is a transmembrane glycoprotein with a single immunoglobulin domain, a cytoplasmic C-terminus rich in proline residues, and a transmembrane domain （Rahimi et al. 2012). Cell adhesion molecules commonly use dimerization to establish their functions. The same is true for IGPR-1. Data suggest that IGPR-1 undergoes covalent cis-dimerization in both extracellular and cytoplasmic domains (Wang et al. 2016). In addition, trans-homophilic dimerization with neighboring cells triggers phosphorylation of IGPR-1 at cytoplasmic region, which is critical for the activation of IGPR-1 (Wang et al. 2016). IGPR-1 also undergoes glycosylation at its extracellular domain. Further

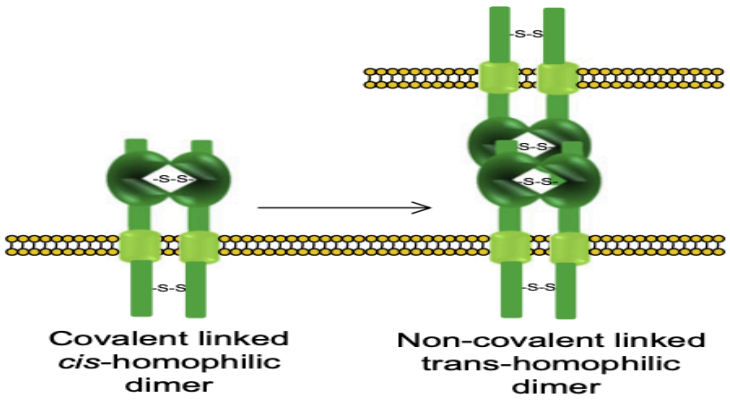


Figure 2. **IGPR-1 forms trans-homophilic dimers by interacting with cis-homodimer IGPR-1 on the opposing cells (Wang et al. 2016).**

research suggested that the highly glycosylated IGPR-1 is necessary for its stability (Rahimi et al. 2012).

The possible role of IGPR-1 at adherence junctions in endothelial cells is that it regulates cell-cell adhesion, cell migration, and angiogenesis (Rahimi et al., 2012). To be more precise, the expression of IGPR-1 inhibits cell migration, and this phenomenon, in part, might be associate with paxillin since the phosphorylation of paxillin is related to inhibition of cell migration (Abou Zeid et al., 2006; Zaidel-Bar et al., 2007; Rahimi et al., 2012). Another vital aspect of IGPR-1 is that it plays a critical role in angiogenesis. Experiments indicate that porcine aortic cells (PAE) overexpressing IGPR-1 undergo increased capillary tube formation (Rahimi et al., 2012). Further investigation also suggests that IGPR-1 contributes to vascular permeability and endothelial barrier function. As mentioned above, trans-homophilic dimerization of IGPR-1 leads to the phosphorylation at serine 220 site on IGPR-1 expressing in porcine aortic cells (PAE) and the Ser220 mutation significantly inhibits the ability of IGPR-1 to direct trans-endothelial electrical resistance (TEER) (Wang et al. 2016). These experiments indicate that IGPR-1 may be essential in some pathological conditions, including inflammation, tumor metastasis, and diabetes caused by impaired endothelial cell-cell adhesion and barrier function of endothelial cells (Wang et al. 2016). Moreover, potential function of IGPR-1 in cancer is not

limited to regulation of endothelial cell function. For instance, IGPR-1 expression is increased in human primary colon cancer, and it promotes colon cancer cell growth in mouse tumor xenograft model. Additionally, IGPR-1 adhesive functions support tumor multicellular aggregation, which increases colorectal tumors' resistance to chemotherapeutic drugs (Woolf et al., 2017).

Protein interactions in order to regulate cellular behavior are commonly accomplished through the recognition of short peptide sequences by modular interaction domains (Li 2005). Both the extracellular and the intracellular domains of IGPR-1 are irreplaceable for this modular interaction. For instance, the Ig-containing domain of IGPR-1 is necessary for its barrier function. Meanwhile, SH3 (Src homology 3) domain and WW domain both recognize the proline-rich sequences (PRDs) at the cytoplasmic region of IGPR-1 (Zarrinpar et al. n.d.; Li 2005; Rahimi 2017). Several studies have indicated that the proline-rich domain of IGPR-1 associates with multiple SH3 domain-containing proteins such as bullous pemphigoid antigen 1 (BPAG1), SPIN90/WISH (SH3 protein interacting with Nck), melanoma inhibitory activity (MIA), and calcium channel β2 subunit (CACNB2) (Rahimi et al.,

2012).

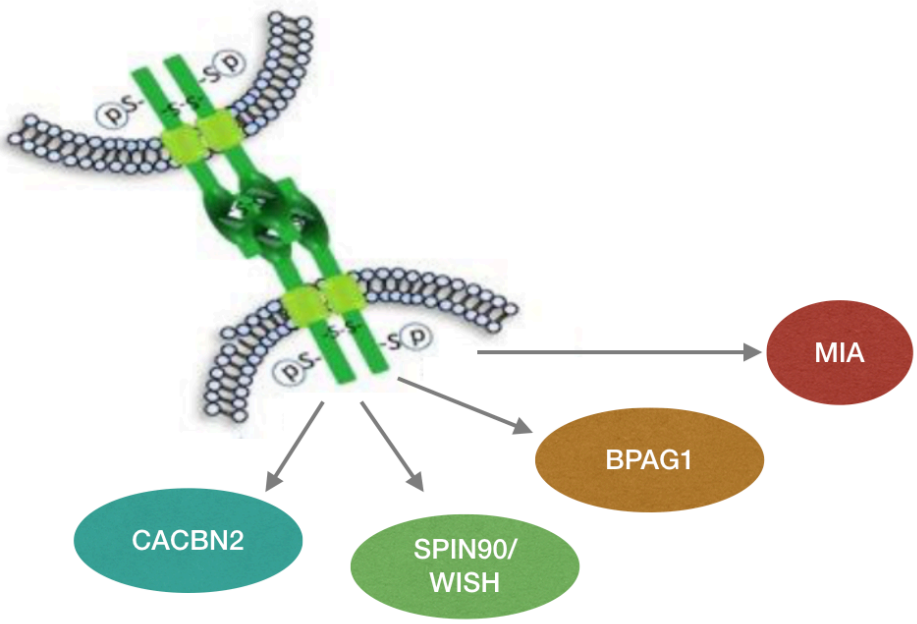


Figure 3. **Cytoplasmic SH3-containing signaling proteins interacting with IGPR-1**

SPIN90(SH3 protein interacting with Nck), also known as WISH or DIP, has been proved to participate in actin polymerization through binding to both the G-actin and Arp2/3 complex (actin-related proteins Arp 2 and Arp 3) (Kim et al. 2006). In addition，SPIN90 interacts with mDia, a Rho GTPase effector protein, and participates in the formation of stress fibers (Satoh and Tominaga 2001). Furthermore, SPIN90 / WISH and PSD-95 are co-localized in dendritic spines and modulate dendritic spinogenesis (Lee et al. 2006). It is interesting to note that SPIN90 associating with IGPR-1 is required for IGPR-1 mediated angiogenesis. Over-

expression of SPIN90 proteins in PAE cells increases the capillary tube formation significantly, suggesting that SPIN90 associates with IGPR-1 via its SH3 domain to modulate angiogenesis (Rahimi et al., 2012). bullous pemphigoid antigen 1 (BPAG1) is also a SH3 domain containing protein that exhibits complicated functions in various organs, including epidermis, nervous system, skeletal muscle, and cardiac muscle (Künzli et al. 2016). With the help of microtubules and various vesicle transmembrane proteins including clathrin, retrolinkin, and dynactin, BPAG1 plays a vital role in both anterograde and retrograde transport, furthermore, it also participates in maintaining membrane-related complexes (Ali et al. 2017). Mutations that affect BPAG1 isoform (BPAG1e) might result in an autosomal dominant or recessive blistering skin disease, bullous epidermolysis (EB) (Groves et al. 2010; Liu et al. 2012). Additionally, BPAG1 also affects neurological diseases. Several studies have shown that genetic mutations of BPAG1 are one of the causes of a devastating neurological disease called hereditary sensory autonomic neuropathy type VI (HSAN-VI) (Künzli et al. 2016). CACNB2, on the other hand, is one of the L-type voltage-pendent calcium channels that mediate calcium entry into cells (Yamagata and Sanes 2008). In fact, CACNB2 plays an important role in cardiovascular disease. In addition, several genes including CACNB2 are contributed to the regulation of blood pressure (Liu et al. 2012). Through the study of three independent models, it was

found that the expression of CACNB2 and the activity of the single ventricle L-type Ca2+ -channels (L-VDCC), a marker of human heart failure, increase simultaneously, which proves the interrelationships between the expression of CACNB2 and human heart failure (Hullin and Matthes 2007). Taken together, given the role of CACNB2 in cardiovascular disease and the fact that CACNB2 interacts with IGPR-1, provide us a new insight of the potential role of IGPR-1 in cardiovascular system and a possible therapeutic implication in such diseases.

Another major known protein domain that interacts with PRDs is the WW domain as we mentioned above.

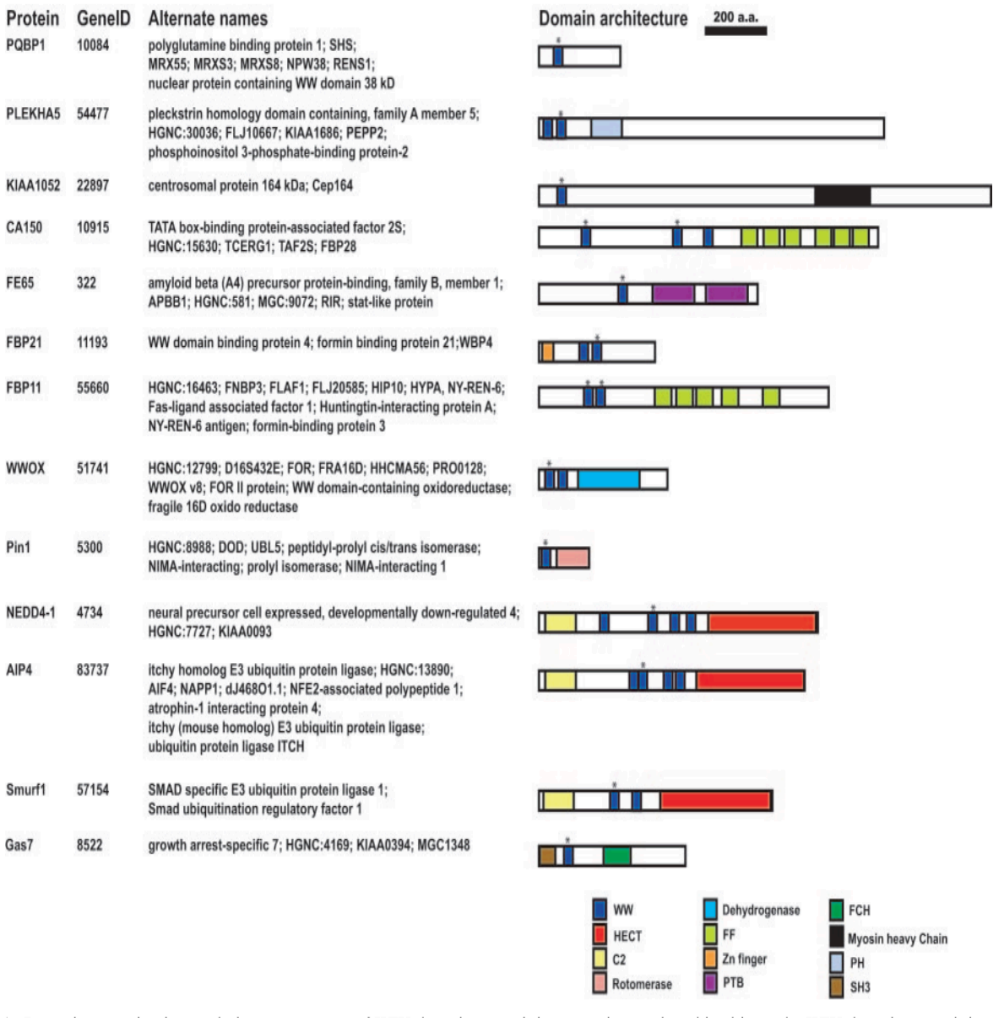


Figure 4. **IDENTIFICATION OF WW DOMAIN-BINDING PROTEINS (Ingham et al. 2005)**

Studies have identified 148 human-related proteins containing WW domains, of which WW domains mediate protein-protein interaction by recognizing phosphorylated serine/threonine-proline and proline rich sequences (Ingham et al. 2005).

The WW domain consists of only 40 amino acids (Chen and Sudol 1995), named after the presence of two highly conserved tryptophan (W) within the sequences, and it is worth mentioning that the WW domain is folded into a meandering triple-stranded antiparallel β-sheet (Macias et al. 1996). Several experiments have demonstrated that the interaction between WW domain and the PRDs could be modulated by tyrosine phosphorylation (Macias et al. 1996) (James n.d.). Interestingly, researchers reported that WW domain interactions may be disrupted by the phosphorylation at the tyrosine residue in the proline containing motif (Chen et al. 1997). Consequently, WW domain containing proteins regulate a variety of cellular processes in different tissues, including RNA synthesis, transcription, (Sudol et al. 2001), protein degradation (Ingham et al. 2004), receptor signal transduction, and regulation of the cytoskeleton (Ilsley et al. 2002). For example, NEDD4 ubiquitin protein ligase, is reported to bind to the α subunit of the Na+ channel through its WW domain and disrupts the activity of epithelial Na+ channel， affecting the salt and water homeostasis (Farr et al. 2000). Moreover, Proteins with WW domains are also located in nucleus. Among them, the WW domain containing protein, Yes Kinase-Associated Protein (YAP), acts as a co-activator of transcription by the protein-protein interaction with Polyoma Enhancer Binding Protein 2 (PEBP2) (Sudol et al. 2001). Another example is about β- dystroglycan, a transmembrane protein

containing proline-rich sequences that bind to the WW domains of the cytoskeleton proteins dystrophin and utrophin, which in turn interact with the actin cytoskeleton (Ilsley et al. 2002). Interestingly, Caveolin-3 also contains a similar WW domain causing competition with dystrophin and utrophin for binding to β-dystroglycan (Sotgia et al. 2000).

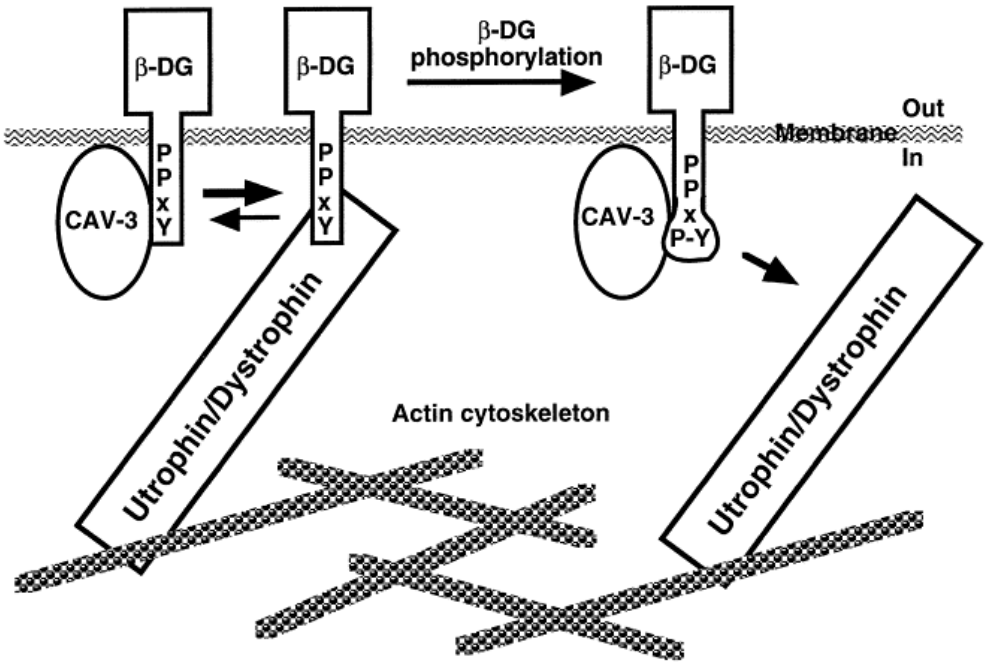


Figure 5. **Differential binding of caveolin-3 or dystrophin to b-dystroglycan is regulated by tyrosine phosphorylation (Ilsley et al. 2002)**

The function of WW domain containing proteins is also linked to the progression of cancer (Bednarek et al. N.d.) (Wulf 2001). Research have identified WW domain-containing oxidoreductase (WWOX) as a tumor suppressor. Downregulation of WWOX protein is observed in majority of invasive cancer, such as breast cancer

(Jamous and Salah 2018) (Chang et al. 2007). Furthermore, WW domain is also involved in genetic diseases. The WW domain containing protein, NEDD4, affects Liddle syndrome by inhibiting epithelial Na + channel (Staub et al. 1996). WW domain containing proteins with ubiquitin E3 ligase activity can mediate protein-protein interactions leading to protein degradation of non-receptor signaling or pre-mRNA splicing proteins, which may involve in Huntington's disease (Faber 1998) (Passani 2000), and the WW domain of prolyl isomerase Pin1 is essential in Alzheimer's disease（Liou, Y., Sun, A., Ryo, A. et al. 2003）（Lu, P., Wulf, G., Zhou, X. et al. 1999）.

Ubiquitination

Ubiquitination is a reversible post-translational modification of proteins that plays a central role in many cellular processes, such as protein degradation, cell cycle regulation, and regulation of apoptosis. The process of ubiquitination is accomplished by ubiquitin. Ubiquitin, a highly conserved protein widely expressed in eukaryotes, consists of 76 amino acids. Ubiquitin has seven lysine residues, and all are involved in ubiquitination. The majority of the lysine 48 linked polyubiquitination is related with the degradation of substrates by proteasome, whereas lysine 63 linked chains are usually involved in non-proteolytic functions (Bedford et al. 2010). Ubiquitin is covalently attached to the target protein, and this process is catalyzed by three

substrate-recruiting enzymes (E1, E2, and E3) (Kimura and Tanaka 2010) (Bloom et al. 2003). The connection of ubiquitin to the substrate involves three steps: ubiquitin-activating enzyme, E1, activates the C-terminus of the ubiquitin protein in an ATP-dependent manner so that it could eventually bind to the amino group of the substrate (Pickart and Eddins 2004). Then ubiquitin is transferred to the ubiquitin-conjugating enzyme (E2). Finally, ubiquitin protein ligase (E3) binds ubiquitin molecules to the substrate via the amino group of a lysine residue (Rahimi 2012). Ubiquitin-recruiting components of the ubiquitin-proteasome system (UPS) continue to catalyze ubiquitin, causing repeated conjugation of ubiquitin moieties to each other to generate a polyubiquitin chain, acting as a binding and degradation signal for 26S proteasome (Rahimi 2012). 26S proteasome complex contains a 20S proteolytic core particle (CP) and a 19S regulatory particle (RP) (Glickman et al. 1998). The 19S complex has two subunits, base and lid. The base is composed of two non-ATPase subunits and six ATPases. Meanwhile, the lid is composed of up to 10 non-ATPase subunits. 19S selects ubiquitinated substrates for translocation into CP. The CP complex consists of 4 heptamers, of which two outer rings contain the α subunits and two inner rings contain the β subunits. Studies have shown that the function of subunits β1, β2, and β5 are post-glutamyl peptide hydrolyzing, trypsin-like, and chymotrypsin-like cleavage respectively (Rahimi 2012).

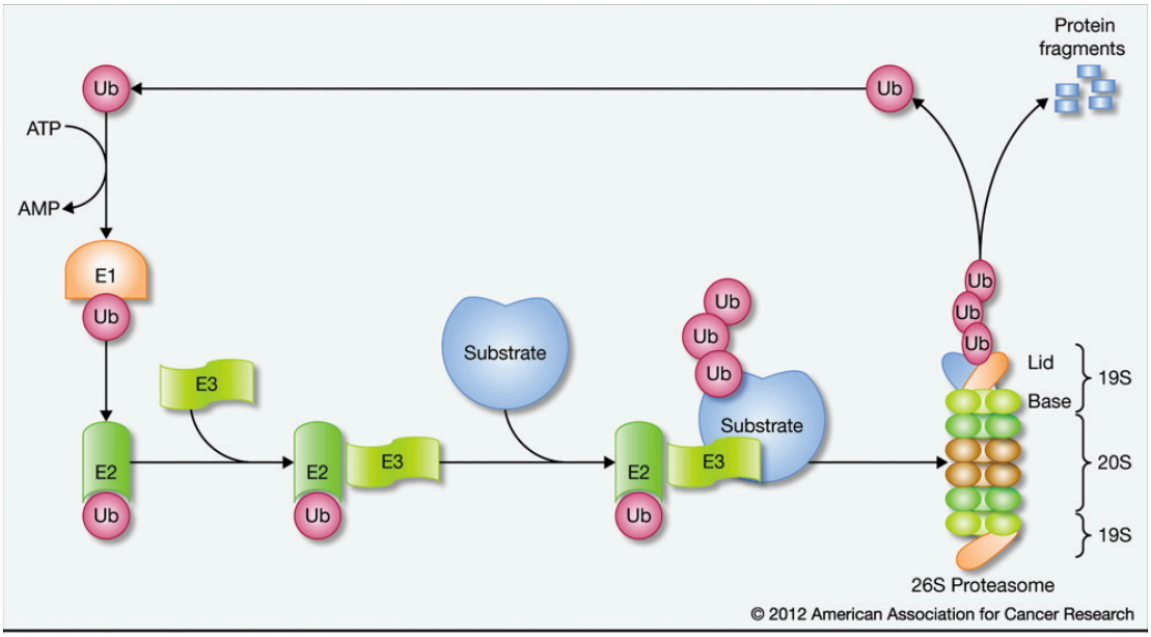


Figure 6. **Schematic of Ubiquitination (Rahimi 2012)**

26S proteasome could recognize target proteins with high specificity although it only interacts with one single targeting signal (polyubiquitin chain). This is because of the variety of E3 ubiquitin ligases that can recognize one or few unique substrates (Pickart and Cohen 2004) (Bedford et al. 2010). E3 ubiquitin ligases are divided into two groups: The Really Interesting New Gene (RING) domain-containing E3 ligases and the homology to the E6-associated protein carboxyl terminus (HECT) domain-containing E3 ligases (Rahimi 2012). The first kind of E3 (RING domain containing E3 ligases) acts as a scaffold that brings together the substrate and E2 enzyme, enabling activated ubiquitin transferring from E2 to the substrate. And in another case, E3 (HECT domain containing E3 ligases) serves a catalytic role to activate

ubiquitin before transferring it from E2 to E3 and then transfer to the substrate (Glickman and Ciechanover 2002). The RING domain is defined by a pattern of conserved cysteine and histidine residues and adopts a unique arrangement to combine two zinc atoms. In this arrangement, zinc 1 is associated with the first and third pairs of zinc ligands, and the second share zinc 2 with the fourth pair of ligands. This zinc linkage is necessary for the RING domain folding and the subsequent biological effects (Borden n.d.). HECT domain proteins contain a 350-amino acid residue homologous to the C-terminal domain of the E6-AP (E6- associated protein) family. Furthermore, HECT domain has a conserved cysteine residue. The activated ubiquitin moiety is usually transferred from E2 to this residue (Scheffner, M., Nuber, U. & Huibregtse, J. 1995) (Huibregtse nd). Interestingly, changes in the N-terminal domain of HECT domain proteins may be related to specific substrate recognition (Glickman and Ciechanover 2002).

Ubiquitin E3 ligase-NEDD-4

Neural precursor cell expressed developmentally down regulated 4 (NEDD4), is one of the first HECT domain containing E3 ligases discovered. Subsequently, several other human HECT E3 ubiquitin ligases were discovered, including three families. Among the HECT family proteins, NEDD4 and HERC families have unique domain architectures, and the remaining HECT ligases contain variable numbers and

types of domains (Scheffner and Kumar 2014). Many researchers have found that NEDD4-like proteins are expressed in eukaryotes, such as yeast and drosophila with similar domains and structures (Ingham et al. 2004). All NEDD4 family proteins share the same structural module, including the C-terminal HECT domain, 3-4 WW domains and the N-terminal C2 domain (Boase and Kumar 2015)

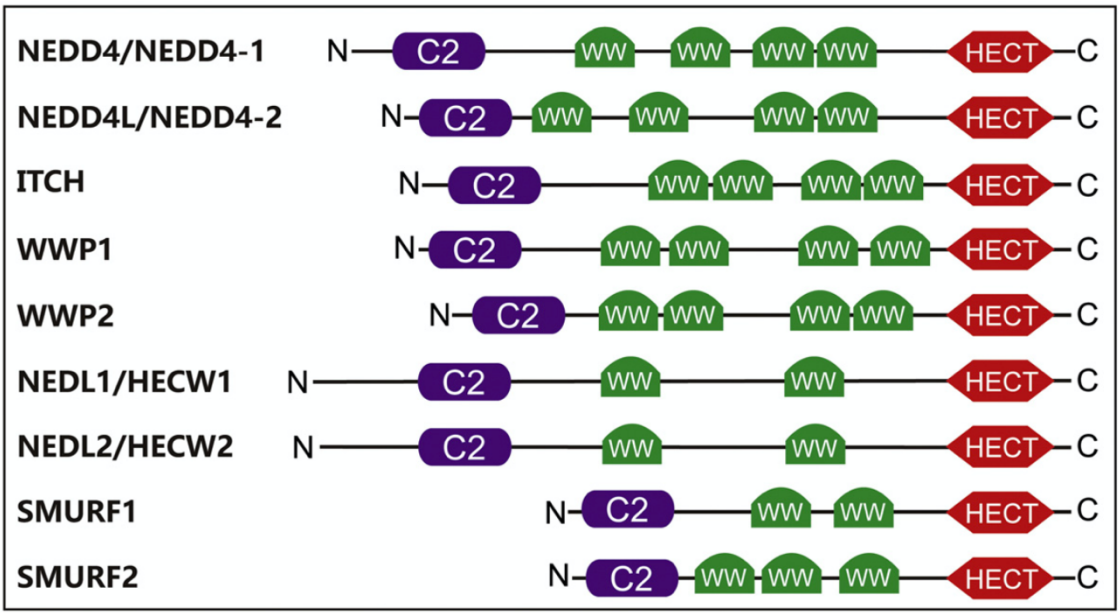


Figure 7. **Schematic diagram of functional domains of the nine members of human NEDD4 E3s (Zou et al. 2015)**

As mentioned above, the HECT domain is involved in the ubiquitination process, whereas protein-protein interaction domains, WW domains, are able to interact with proline rice domains. C2 domain consists of around 120 amino acids binding to the cell membrane in a Ca2+ dependent manner via electrostatic interaction between negatively charged lipids and basic amino acids (Cho 2001) (Dunn et al. 2004). C2 mediated protein-lipid binding is important for several

functions of NEDD4 (Dunn et al. 2004). Numerous approaches are used to identify the substrates and the potential function of NEDD4.

The amiloride-sensitive epithelial sodium channel (ENaC) is a transmembrane ion channel that is essential in sodium transport. ENaC contains two proline rich sequences in its C-terminus. Yeast two hybrid studies have shown that NEDD4 is associated with the proline-rich sequences of ENaC through its WW domain, indicating that NEDD4 is a regulator of the epithelial Na+ channel (Staub et al. 1996). NEDD4 is also involved in modulating Alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid receptors (AMPAR), a major mediator of excitatory synaptic transmission. It has been reported that NEDD4 ubiquitylated mammalian AMPARs in neurons and transfected heterologous cells, resulting in significant downregulation of AMPARs at the cell surface and also causing a reduction in synaptic transmission (Lin et al. 2011). Furthermore, the voltage-gated calcium ion channels are also influenced by NEDD4. Surface biotinylation assays and SDS-PAGE revealed that NEDD4 induces the downregulation of calcium ion channels at the plasma membrane (Rougier et al. 2011). NEDD4 also plays a prominent role in the epidermal growth factor (EGF) signaling pathway (Boase and Kumar 2015). Firstly, type I transmembrane receptor tyrosine kinase (ErbB4), a member of the epidermal growth factor receptor (EGFR) family, is degraded following ubiquitination in a NEDD4-

dependent manner (Zeng et al. 2009). Moreover, the proline-rich sequence of the activated Cdc42-associated tyrosine kinase (ACK) binds to the WW3 domain of NEDD4, leading to the ubiquitination and degradation of ACK (Lin et al. 2010). NEDD4 also regulates endocytosis and signal transduction of fibroblast growth factor receptor 1 (FGFR1) through ubiquitination. Interestingly, WW domain of NEDD4 binds to FGFR1 through a novel non-canonical sequence (non-PY motif) on FGFR1, and deletion of this recognition motif (FGFR1-D6) impairs the binding of NEDD4 as well as its function (Persaud et al. 2011).

Overexpression of NEDD4 is commonly observed in many types of cancer, including colorectal, breast, gastric, and non-small cell lung cancer (Zou et al. 2015). While the molecular explanations for tumor-promoting role of NEDD4 remain to be explored, scientists have given some potential reasons. Studies have shown that a subunit of the class III phosphatidylinositol 3 kinase complex, also known as Beclin1, is a tumor suppressor. The PY motif of Beclin1 binds to the NEDD4 WW domain, causing the polyubiquitination and subsequent downregulation of Beclin1 (Platta et al., 2012). Another study explores the relationship between NEDD4 and a tumor suppressor, called Phosphatase and tensin homolog (PTEN). Mutations and deletions of PTEN frequently occur in various human cancers, and NEDD4 negatively regulates its expression by catalyzing PTEN polyubiquitination. In addition, the use

of mouse cancer models or human cancer samples has demonstrated that low expression of PTEN is co-localized with high expression of NEDD4. Therefore, NEDD4 may be a potential proto-oncogene (Wang et al. 2007). Interestingly, the ubiquitin-mediated process involves not only in protein degradation through the 26S proteasome pathway but also in biosynthesis and endocytosis (Ingham et al. 2004). PTEN is a good example. NEDD4 monoubiquitinates PTEN mainly on lys13 and lys289, thereby promoting its nuclear translocation (Trotman et al. 2007). Previous research highlights that PTEN suppresses tumorigenesis in the nucleus by mediating DNA damage repair to guard the chromosomal integrity. (Shen et al. 2007) (Choi et al. 2013) (Bassi et al. 2013). These data explain that NEDD4 regulates PTEN expression both positively and negatively. Downregulation of NEDD4 expression can also be associated with carcinogenesis by regulating Myc oncoprotein. It has been demonstrated that class III histone deacetylase SIRT2 is increased by N-Myc in neuroblastoma cells and by c-Myc in pancreatic cancer cells. Meanwhile, SIRT2 raises the stability of N-Myc and c-Myc proteins, leading to the proliferation of cancer cells. However, NEDD4 significantly inhibits SIRT2 through ubiquitination, and more importantly, NEDD4 directly targets Myc oncoprotein, making it ubiquitinated and degraded (Liu et al. 2013). These data fully illustrate the

significance of NEDD4 in tumorigenesis and provide the potential therapeutic targets for future studies.

Interestingly, many viruses use NEDD4 to facilitate their budding, including Ebola virus, Human immunodeficiency virus (HIV), Moloney murine leukemia virus, Mason-Pfizer monkey virus, Rous sarcoma virus. They all contain the PY late budding motifs, which is pivotal for virion budding and release (Ingham et al. 2004) (Boase and Kumar 2015).

Overall, both NEDD4 and IGPR-1 are precisely regulated by various factors to accomplish their unique functions. As mentioned earlier, proline-rich domains of IGPR-1 could interact with both WW and SH3 domains. The overall goal of this thesis project is to investigate the potential of IGPR-1 to interact with WW domain containing ubiquitin E3 ligase, NEDD4 and explore possible mechanism involved.

SPECIFIC AIMS AND OBJECTIVES

IGPR-1 is expressed in endothelial cells and regulates angiogenesis and endothelial cell barrier function. Furthermore, IGPR-1 is highly expressed in human colon cancer cells, and the over-expressing of IGPR-1 stimulates tumor growth and plays major role in endothelial cell function (Woolf et al., 2017). However, the molecular mechanisms governing expression of IGPR-1 remains unknown. The overall goals of this project are to test the hypothesis that IGPR-1 interacts with

NEDD4 involving WW domains of NEDD4 and the proline rich motif of IGPR-1, which leads to ubiquitination of IGPR-1. The overall specific aims of this project are:

Specific aim I: Test the hypothesis that NEDD4 binds to IGPR-1 and that a specific WW domain of NEDD4 mediates the binding of NEDD4 with IGPR-1

Specific aim II: Test the hypothesis that NEDD4 binds to and ubiquitinates IGPR-1 leading to its downregulation.

Specific aim III: Identify potential NEDD4-binding ubiquitin conjugating ubiquitin E2 ligases involved in the ubiquitination of IGPR-1

METHODS

Cell Culture

Human Embryonic Kidney cells (HEK-293) were grown and maintained in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (FBS). Cells were placed in a humidified incubator at 37°C and 5% CO2. Cells were sub-cultured by using trypsin/EDTA regularly and they were free of contamination.

Antibodies and other Reagents

Rabbit polyclonal antibody against IGPR-1 was used in 1:1000 dilution in western blotting to detect the presence of IGPR-1. Anti-pSer220 antibody was used in 1:1000 dilution to detect the Ser220 phosphorylation of IGPR-1. The mouse monoclonal anti-HA and anti-Myc antibody were used for western blot analysis and the detection of the IP products of the HA- and Myc- tagged proteins.

Cell Transfection

HEK-293 cells were grown in DMEM 10% FBS media to 80% confluency. Prior to transfection, media was removed, and 2 mL of serum free DMEM was added to each 60 mm cell culture dish. Then, DNA-PEI (polyethyleneimine) mixtures were prepared in tissue culture hood containing 500ml of serum free DMEM, 9µl of (PEI)

and 3µg of DNA from pcDNA3.1 His/Myc, pcDNA HA-NEDD4 wild type, and pcDNA Myc-ΔWW-NEDD4 plasmids. Following incubation for 15 minutes, mixtures were applied onto cells and plates were returned to 37°C incubator. After 6 hours, 2mL of DMEM 10% FBS was added to plates and the next day, existing media (2 mL of serum free DMEM plus 2mL of DMEM 10% FBS) was replaced with DMEM 10% FBS gently. After 48 hours, transfected cells were lysed and examined by SDS-PAGE.

Western Blot Analysis

HEK-293 cells culture dishes were placed on ice, rinsed twice with chilled H/S buffer [20 mM Hepes (pH 7.4) and 150 mM NaCl] then lysed using lysis (EB) buffer [10mM Tris- HCl (pH 7.5), 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1%Triton X-100] containing 10uL/ml Na3VO4 and 15 ul/ml PIC (Protease Inhibitor Cocktail). Whole cell lysates were centrifuged. Then, 5X sample buffer [3.8% Tris-base, 50% glycerol, 5% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, and .0025% bromophenol blue] was added to collected supernatant and samples were boiled at 95°C for 5 minutes. Cell lysates were loaded on a 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes pre-activated by methanol. PVDF membranes were blocked with Blotto (2% non-fat dry milk and 0.05% Tween-20 in Western Rinse) for 1 hour on a rocker.

Following washing with western rinse for 5 minutes, membranes were immunoblotted with primary antibodies for 1 hour at room temperature. After three washes with western rinse (each time 10 minutes), membranes were incubated with secondary antibodies for 1 hour at room temperature. Following three-times 10 minutes wash with western rinse and adding enhanced chemiluminescence (ECL), and membranes were developed.

Co-immunoprecipitation assay (Co-IP)

Co-IP of expressed proteins was performed using HEK293 cells transfected with pcDNA3.1 His/Myc and pcDNA HA-NEDD4 wild type. After 48 hours, cells were harvested using lysis buffer. Lysates were then incubated 4hours with 5µl anti-IGPR-1 antibody and then 1 hour with Protein A beads. The resulting complexes were washed with PBS containing PIC (15µl/ml) three times, eluted with 2X sample buffer, and boiled at 95°C for 5 minutes. Final products were examined using SDS-PAGE.

Recombinant GST fusion protein production and GST pull-down assay

GST fusion proteins were expressed in E. coli strain BL21. The E. coli cells were harvested by centrifugation and then disrupted by sonication. The supernatants of the cell lysates were subjected to a glutathione Sepharose 4B affinity column to purify the GST fused IGPR-1, NEDD4, NEDD4 WW domain 1,3, and 4. The quality of purified proteins was analyzed by SDS-PAGE. The purified proteins were

subsequently used for GST-pull down assay. Purified proteins incubated with whole cell lysates for 4 hours, then washed with PBS containing PIC (15µl/ml) three times, eluted with 2X sample buffer, and boiled at 95°C for 5 minutes. Final products were examined using SDS-PAGE.

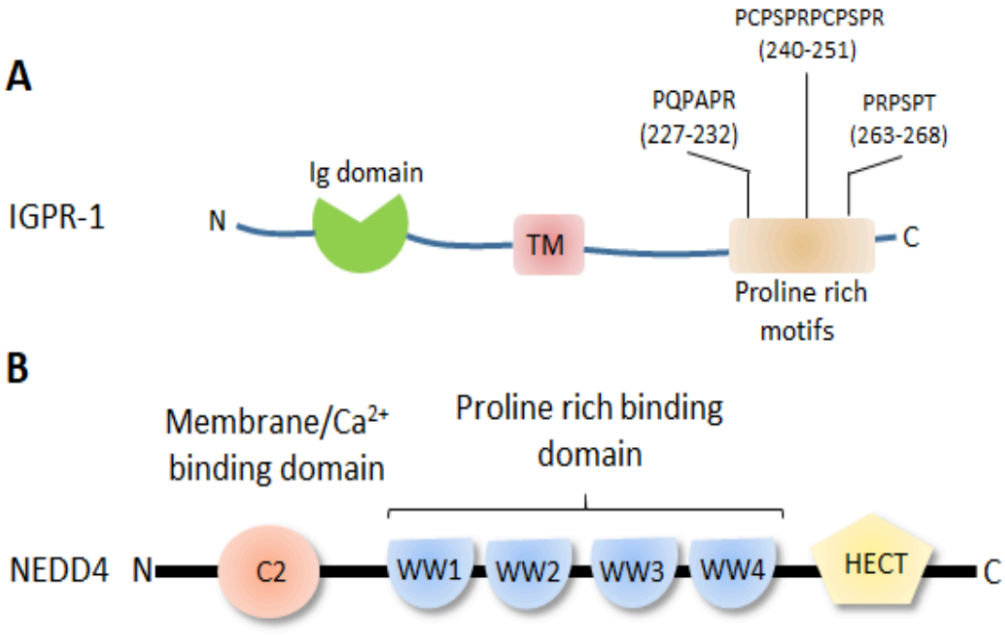
In vitro Ubiquitination Assay

In vitro ubiquitination assay kit was purchased from Endo company and it was performed following the protocols provided by the manufacturer. For each sample, the reaction was performed in total 50 µl, including 14µl ddH2O (double distilled water), 5µl ubiquitination buffer, 10µl IPP (Inorganic pyrophosphatase solution, 100 U/mL in 20 mM Tris-Cl, pH 7.5), 1µl DTT ((50 mM in 20 mM tris-Cl, pH 7.5), 2.5µl Mg-ATP (0.1M), 2.5µl E1 (2µM), 5µl E2 (25µM), 2.5 µl biotinylated ubiquitin (50µM), 2.5µl E3 (GST-NEDD4) and 5µl target protein (GST-IGPR-1). Samples were gently mixed and incubated for 2 hours at 37°C. 50 µL of 2X non-reducing gel loading buffer was added before analyzing by western blot.

RESULTS

IGPR-1 interacts with NEDD-4

To test our hypothesis that IGPR-1 binds to NEDD4, we carried out a co-immunoprecipitation assay (Co-IP) on HEK 293 cells expressing IGPR-1 or empty vector (EV). pcDNA3.1-His/Myc vector and pcDNA HA-NEDD4 wild type plasmids were transient transfected into EV/HEK 293 cells and IGPR-1/HEK 293 cells. Cell lysates were then subjected to immunoprecipitation with an anti-IGPR-1 antibody. Followed by western blots analysis using antibodies corresponding to the Co-IP products and inputs revealed that NEDD4 binds to IGPR-1 (Figure 8).



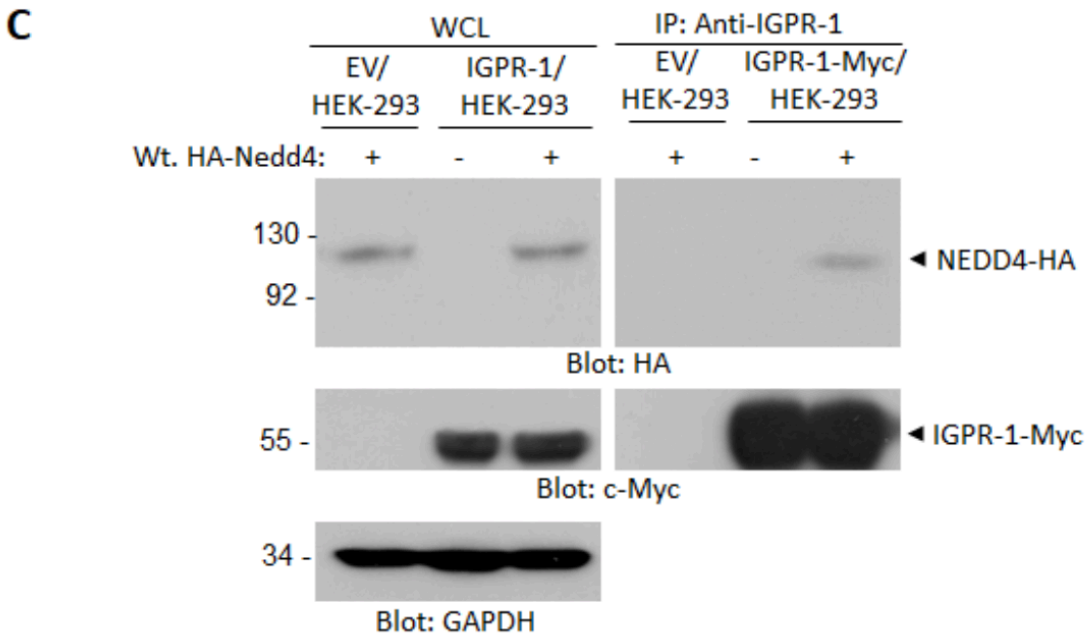
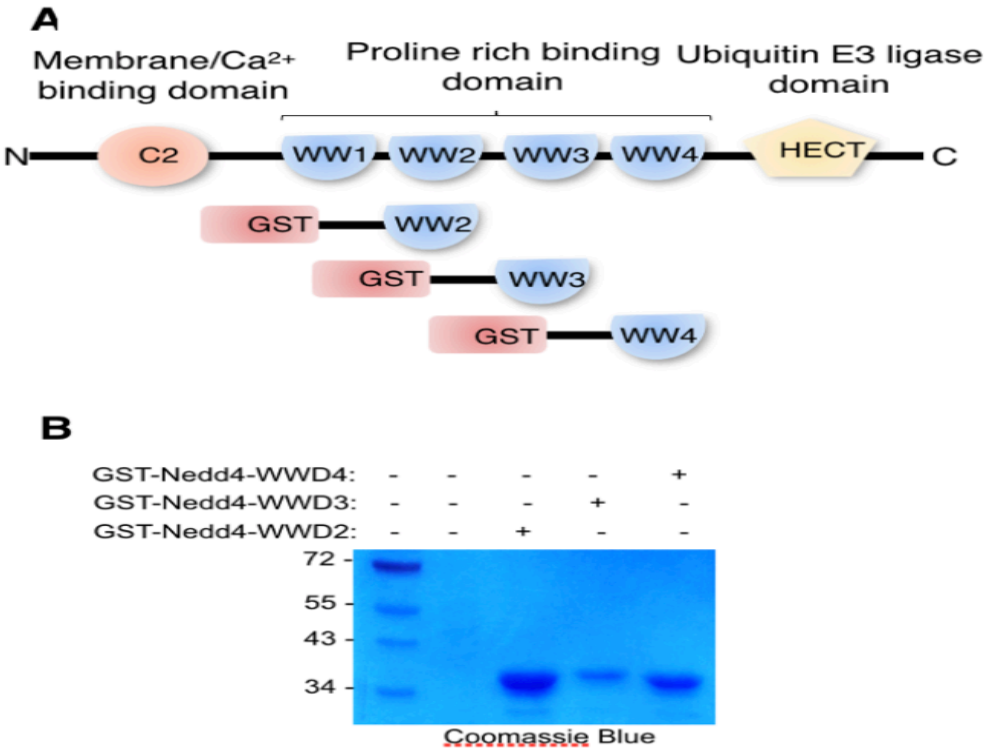


Figure 8. **NEDD4 bands to IGPR-1 in vitro.** (A) Diagram of the function domain of IGPR-1. (B) Diagram of the function domain of NEDD4. (C) Cells were lysed and subjected to western blot analysis using the anti-Myc antibody, anti-HA antibody, and anti-GAPDH antibody for protein loading control. Co-IPed proteins were assayed using the anti-Myc antibody and anti-HA antibody.

NEDD4 binds to IGPR-1 through its WW4 domain

To further evaluate the interaction of NEDD4 with IGPR-1, we expressed and purified the following recombinant proteins: GST-NEDD4-WW domain 2, GST-NEDD4-WW domain 3, GST-NEDD4-WW domain 4 (Figure 9B). Then, we performed GST pull-down assay by incubating the HEK293-IGPR-1 cell lysates with the recombinant proteins respectively. Data demonstrated that Myc-tagged IGPR-1

could be pulled down by the NEDD4-WW domain 4, indicating that NEDD4 directly binds to IGPR-1 via its WW4 domain (Figure 9).



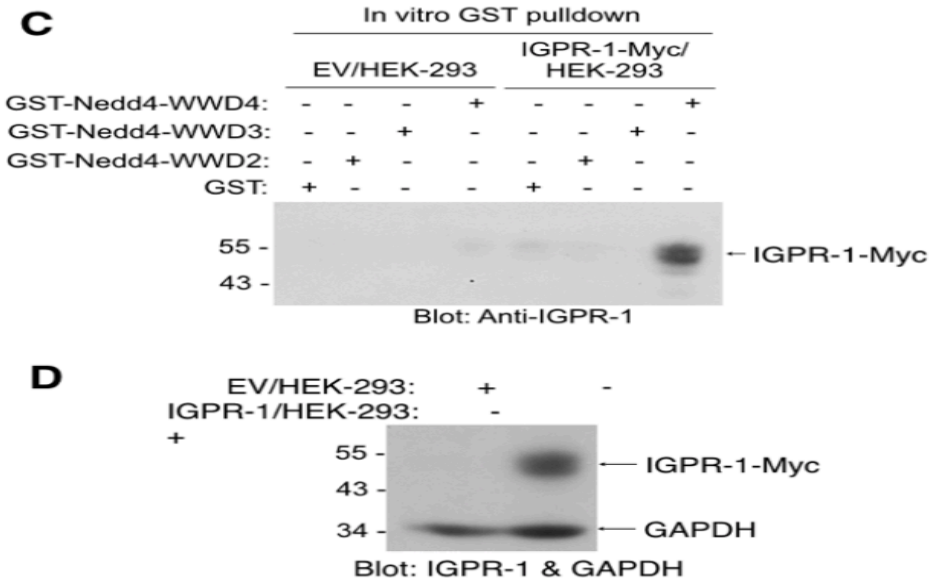
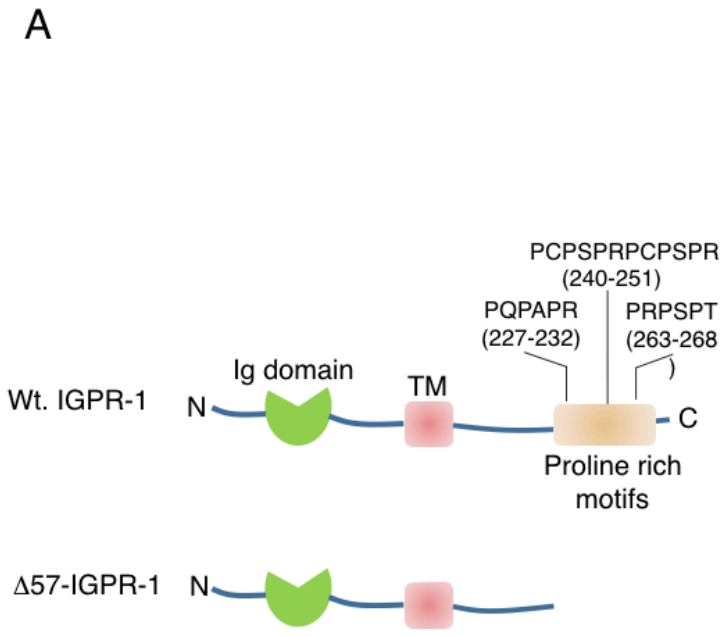


Figure 9. **NEDD4 directly binds to IGPR-1 via its WW4 domain.** (A) schematic diagram of the recombinant proteins. (B) purified GST-fused NEDD4 WW domain 2, domain 3, and domain 4 proteins were analyzed by SDA-PAGE and Coomassie blue staining. (C) The purified proteins were incubated with HEK293-IGPR-1 cell lysates. Then the products were examined by SDS-PAGE and immunoblotting with the anti-IGPR-1 antibody. The HEK293-IGPR-1 cell lysates were immunoblotting with anti-IGPR-1 antibody and anti-GAPDH antibody for loading control.

IGPR-1 binds to NEDD4 through its proline-rich domain

Previous investigation has shown that the WW domain recognizes the proline-rich sequences (PRDs) at the cytoplasmic region of IGPR-1 (Zarrinpar et al. n.d.; Li 2005; Rahimi 2017). Thus, we further determined whether this proline-rich motif is required for the interaction of NEDD4 and IGPR-1. We examined the interaction of

the recombinant proteins, GST-NEDD4-WW domain 4, with HEK293 cells expressing empty vector, wild-type IGPR-1 and proline-rich motif deletion IGPR-1(Δ57-IGPR-1). As shown in figure 10, mutant IGPR-1 were unable to interact with NRDD4, whereas the wild-type IGPR-1 directly binds to NEDD4 WW domain 4 (Figure 10)



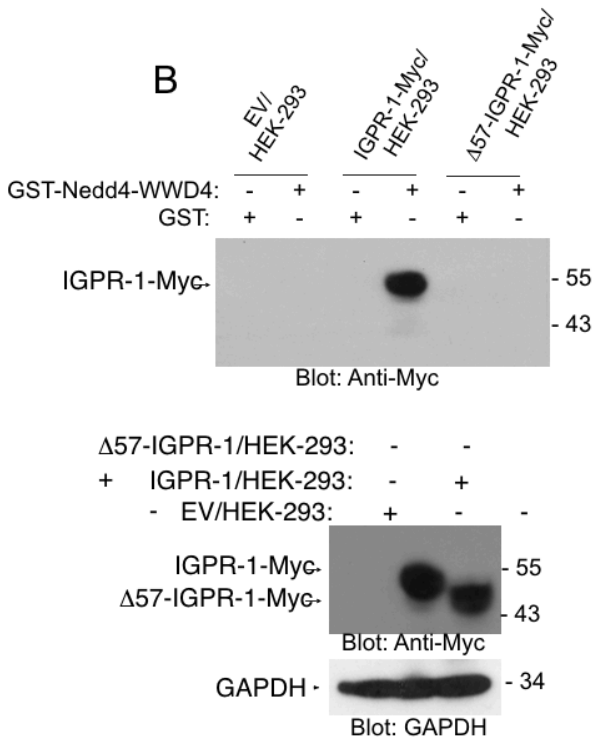


Figure 10. **IGPR-1 directly binds to NEDD4 via its proline rich domain.** (A) Schematic diagram of wild-type IGPR-1 and Δ57-IGPR-1. (B) Purified GST-fused NEDD4 WW domain 4 proteins were incubated with HEK293-EV, HEK293-IGPR-1 and HEK293-Δ57-IGPR-1 cell lysates. Then the products were examined by SDS-PAGE and immunoblotting with anti-Myc antibody. The whole cell lysates were immunoblotting with anti-Myc antibody and anti-GAPDH antibody for loading control.

NEDD4 promotes ubiquitination of IGPR-1

Next, we examined the role of NEDD binding with IGPR-1, in particular whether NEDD4 can promote ubiquitination of IGPR-1. To reveal the potential role of NEDD4, we conducted another Co-immunoprecipitation assay by transfecting pcDNA3.1-His/Myc and pcDNA HA-NEDD4 wild type plasmids into HEK293-

IGPR-1 cell lines. Half of the cells were treated with bortezomib, a drug that interacts with the catalytic site of the 26S proteasome with high affinity and specificity to inhibit protein degradation. All the cells were lysed after 48 hours and IP with IGPR-1, then subjected to western blot analysis. Evidence indicated that NEDD4 induced the poly-ubiquitination of IGPR-1 at both lysine 48 and lysine 63, corresponding to the prior investigation that lysine 48 linked polyubiquitination is associated with degradation of substrates, whereas lysine 63 linked chains are usually related to non-proteolytic functions such as endocytosis (Bedford et al. 2010). In addition, we also observed the aggregation of poly-ubiquitin and the lower expression of IGPR-1 with the treatment of bortezomib. This result demonstrates that IGPR-1 is degraded through the proteasome pathway. Taken together, we could establish a new model of the degradation of IGPR-1, which is poly-ubiquitination at lysine 63 induced endocytosis and lysine 48 induced degradation by proteasome.

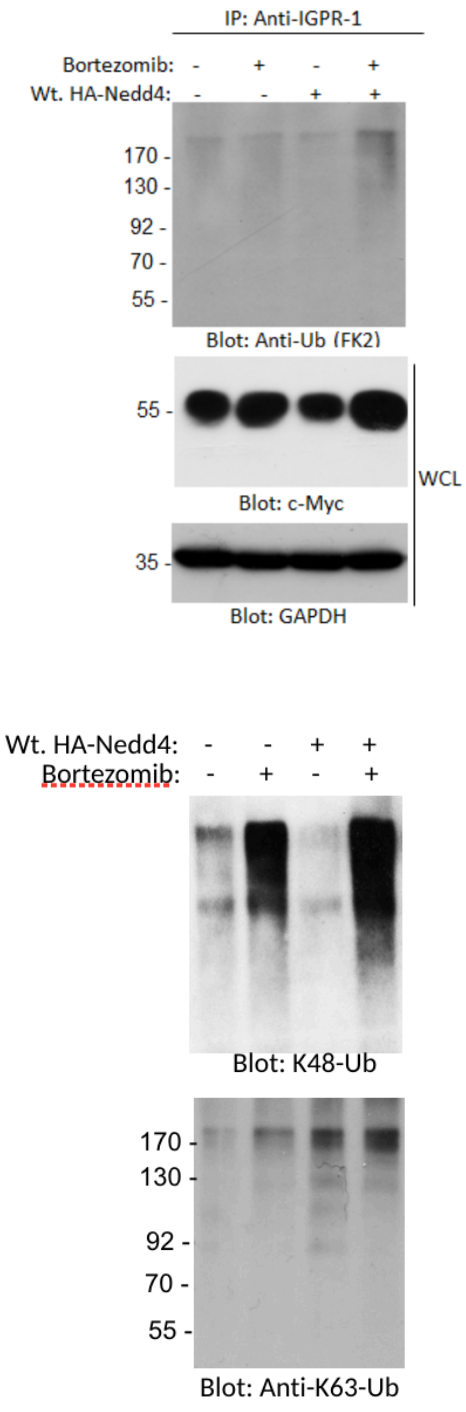


Figure 11. **NEDD4 induces ubiquitination of IGPR-1.** Cells were transfected with pcDNA His/Myc B and pcDNA HA-NEDD4. After 24 hours cells were treated with

or without bortezomib, then lysed after 48 hours and incubated with IGPR-1 antibody for 4 hours. Co-IP products were subjected to western blot analysis using the anti-ubiquitin antibody, anti-K48 antibody, and anti-K63 antibody. Whole cell lysates were analyzed using anti-Myc antibody and anti-GAPDH antibody for loading control.

NEDD4 mediates downregulation of IGPR-1

Next, we decided to examine whether IGPR-1 expression is regulated by NEDD4. To this end, we transfected HEK293-IGPR-1 cells with empty vector, wild-type NEDD4 and the WW domain deletion NEDD4 (ΔWW-NEDD4). Western blot analysis demonstrated that the expression of IGPR-1 as well as phosphorylated IGPR-1 are both significantly reduced in the presence of NEDD4 wild type, suggesting that NEDD4 down-regulates the expression of IGPR-1 (Figure 11).

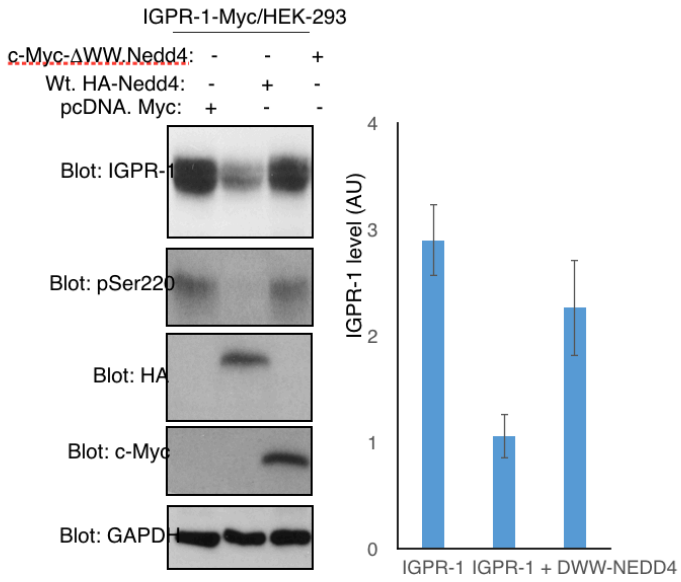
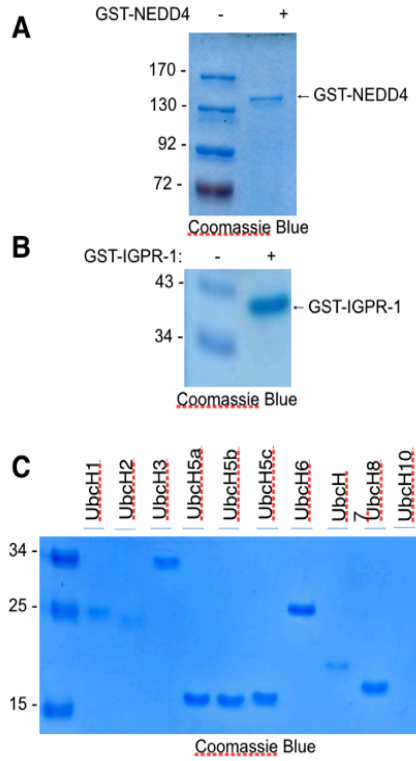


Figure 12. **Expression of IGPR-1 is down-regulated by NEDD4.** (A) HEK293-IGPR-1 cells were transfected with plasmids expressing empty vector, wild-type NEDD4, and WW domain deletion NEDD4 (ΔWW-NEDD4). After 48 hours, lysates of transfected cells were examined by SDS-PAGE and immunoblotted with anti-IGPR-1, anti-pSer220, anti-HA, anti-Myc and anti-GAPDH antibody.

Ubiquitin E2 conjugating UbcH6 is involved in NEDD4 mediated ubiquitination of IGPR-1

To identify putative ubiquitin E2 enzyme involved in NEDD4 mediated ubiquitination of IGPR-1, we used an in vitro ubiquitination assay. Specificity, we tested activity of 11 enzymatically active E2s in vitro ubiquitination assay using recombinant NEDD4 and IGPR-1. Moreover, we analyzed the quality of purified recombinant IGPR-1 to ensure the accuracy of the analysis, NEDD4, and 11 kinds of E2s using SDS-PAGE and coomassie blue staining (Figure 12A, B, C). As shown in Figure 12, in the presence of ubiquitin, GST-NEDD4, GES-IGPR-1, and required other co-factors, we found that E2 enzyme, UbcH6, produced the strongest poly-ubiquitin signal. By contrast, a weak signal was detected in reactions that contain UbcH5a, UbcH5b, UbcH5c, UbcH7, UbcH8, and UbcH13. Comparison of these patterns of the signal indicated that UbcH6 is specific for the NEDD4-dependent ubiquitination of IGPR-1 (Figure 12).


A
GST-NEDD4 - +
170 -
130 -
92 -
72 -
← GST-NEDD4
Coomassie Blue
B
GST-IGPR-1: - +
43 -
34 -
← GST-IGPR-1
Coomassie Blue
C
UbcH1
UbcH2
UbcH3
UbcH5a
UbcH5b
UbcH5c
UbcH6
UbcH7
UbcH8
UbcH10
UbcH13
34 -
25 -
15 -
Coomassie Blue


D
In vitro ubiquitination assay
Biotinylated Ub
+
E2
+
GST-NEDD4
+
GST- IGPR-1
Incubate at 37°C, 2hr

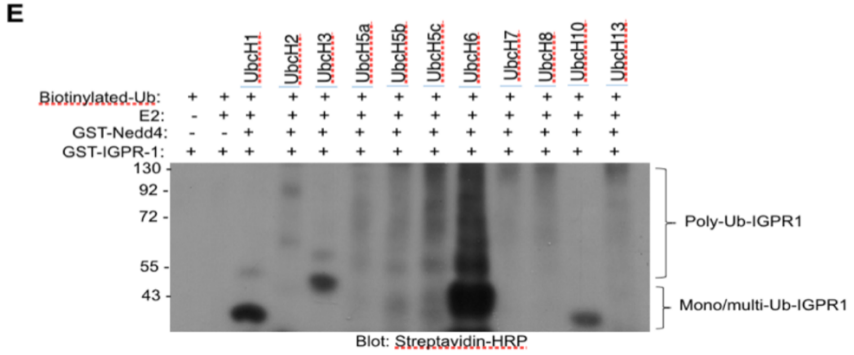

E
UbcH1
UbcH2
UbcH3
UbcH5a
UbcH5b
UbcH5c
UbcH6
UbcH7
UbcH8
UbcH10
UbcH13
Biotinylated-Ub:
E2:
GST-Nedd4:
GST-IGPR-1:
130 -
92 -
72 -
55 -
43 -
Poly-Ub-IGPR1
Mono/multi-Ub-IGPR1
Blot: Streptavidin-HRP

Figure 13. **NEDD4 shows specificity toward E2 enzyme UbcH6.** (A) (B) (C) purified GST-fused NEDD4, IGPR-1 and 11 kinds of E2s were anaylzed by SDA-PAGE and Coomassie blue staining. (D) Diagram of the process of in vitro ubiquitination assay. (E) Products of the in vitro ubiquitination assay were examined by SDS-PAGE using the streptavidin-HRP antibody.

DISCUSSION

IGPR-1 is a novel adhesion molecule, which is widely expressed in endothelial and epithelial cells (Rahimi et al., 2012; Wang et al., 2016). Recent research has shown several critical functions for IGPR-1. It is involved in cell-cell adhesion, endothelial barrier function, and regulation of angiogenesis (Rahimi et al., 2012; Wang et al., 2016).

IGPR-1 expression is upregulated in human primary colon cancer and its activity promotes tumor growth in cell culture and mouse tumor xenograft. More importantly, inhibition of IGPR-1 activity improves the sensitivity of colon cancer cells to the chemotherapeutic agent, doxorubicin (Woolf et al., 2017).

Previous studies identified Src homology domains (SH3) and WW domains containing proteins as IGPR-1 binding partners (Rahimi et al., 2012). In this study, we investigated the role of NEDD4, a WW domain containing E3 ligase, in the regulation of IGPR-1 expression. E3 ubiquitin protein ligase neural precursor cell expressed developmentally down-regulated protein 4, also known as NEDD4, is encoded by the NEDD4 gene (Kumar et al. n.d.). NEDD4 belongs to the E3 HECT ubiquitin ligases family that can regulate numerous proteins expressing on the cell surface, such as membrane receptors and ion channels by ubiquitination and endocytosis (Yang and Kumar 2010) (Scheffner and Kumar 2014). To prove the

interaction of NEDD4 and IGPR-1, we generated HEK293 cell lines expressing IGPR-1 as a model system to study the regulation of IGPR-1 expression.

In this study, we proposed a new model for the NEDD4 function, which is based on its interaction with IGPR-1. To test our hypothesis, we conducted a co-immunoprecipitation assay on HEK293-IGPR-1 cells and demonstrate that NEDD4 interacts with IGPR-1.

Considering the observed direct interaction between IGPR-1 and NEDD4, we sought to elucidate the specific domain involved in the interaction of IGPR-1 and NEDD4. Previous studies have documented that the WW domain recognizes the proline-rich sequences (PRDs) at the cytoplasmic region of IGPR-1 (Zarrinpar et al. n.d.; Li 2005; Rahimi 2017). Results from the pull-down assay demonstrated that IGPR-1 directly bound to NEDD4 in an *in vitro* GST pull down assay and WW domain 4 was required for the interaction. In addition, since the proline rich domain deleted IGPR-1 did not bind to NEDD4, we concluded that proline rich domain is required for interaction of IGPR-1 with NEDD4. This observation is congruent with our hypothesis that WW domain and proline rich domain were involved in the interaction of IGPR-1 and NEDD4.

Another important aspect of our study is the effect of NEDD4 interaction with IGPR-1. NEDD4 is a wildly expressed E3 ligase, raising a possibility that NEDD4

induces ubiquitination and degradation of IGPR-1 through their interaction. In support of this hypothesis, Co-IP assay demonstrated that NEDD4 promoted poly-ubiquitination of IGPR-1 at both lysine 48 and lysine 63. We also observed that the degradation could be inhibited by bortezomib, suggesting that IGPR-1 degraded via the proteasome pathway. Furthermore, decreased IGPR-1 expression has been observed in NEDD4 transfected cell lines. Interestingly, WW domain deletion of NEDD4 did not affect the expression of IGPR-1, which was consistent with previous data about the binding domain of NEDD4. Taken together, our results have shown that NEDD4 binds to IGPR-1 and their interaction leads to the downregulation of IGPR-1 through the proteasome pathway.

To further analyze the mechanism of ubiquitination of IGPR-1 by NEDD4, we set up an *in vitro* ubiquitination assay to identify the E2 conjugating enzyme involving in the pathway. We analyzed 11 different E2s and identified UbcH6 as an important E2 enzyme in the NEDD4-dependent ubiquitination of IGPR-1. Prior investigations also substantiate the belief that specific E2 enzymes such as human Ubc4, UbcH6 and UbcH7, could transfer ubiquitin molecules to NEDD4 at the active cysteine residue (Malakhova and Zhang 2008) (Anan et al. 1998).

It has been demonstrated that IGPR-1 expression is elevated in human colon cancer cells and the over-expression significantly contributes to the development of

resistance to the chemotherapeutic drugs, doxorubicin (Woolf et al., 2017). Our study suggested that NEDD4 regulates expression of IGPR-1, suggesting a novel mechanism for regulation of IGPR-1 expression in endothelial and cancer cells. However, further studies are required to establish the importance of NEDD4 in regulation of IGPR-1 expression. Meanwhile, our data showed the IGPR-1 is degraded via the proteasome pathway, but whether the lysosome pathway is involved in IGPR-1 regulation still remains unknown. Further studies on NEDD4-dependent IGPR-1 ubiquitination and the associated mechanism in vivo could provide new insight into the function and regulation of IGPR-1.

LIST OF ABBREVIATED JOURNAL TITLES

ATVB	Arteriosclerosis, Thrombosis, and Vascular Biology
Cell Commun Signal	Cell Communication and Signaling
Cell Death Differ	Cell Death and Differentiation
EMBO J	EMBO Journal
Exp Dermatol	Experimental Dermatology
Front Immunol	Frontiers in Immunology
Front Oncol	Frontiers in Oncology
Genes Cells	Genes to Cells
J Am Soc Nephrol	Journal of the American Society of Nephrology
J Biol Chem	Journal of Biological Chemistry
Mol Biol Cell	Molecular Biology of the Cell
Nat Rev Immunol	Nature Reviews. Immunology
Nat Rev Mol Cell Biol	Nature Reviews. Molecular Cell Biology
Semin Immunopathol	Seminars in Immunopathology

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