2.1 Materials

2.1.1 Chemicals and reagents

The following chemicals were used in the present study: Ampicillin, EDTA (USB), dNTPs, Taq DNA polymerase (Fermentas), Pfu DNA polymerase (Stratagene), DpnI (New England Biolabs), Plasmid miniprep, midiprep, and maxiprep kits (Qiagen, and Invitrogen), glycine, EGTA, NaCl, Tris (Fisher Scientific), NH4Cl, acrylamide (SRL), Cisplatin, Doxorubicin, MG132, Cadmium chloride, Nonident P-40, propidium iodide (PI), bis-acrylamide, SDS, TEMED, Ammonium persulphate (APS), Coomassie Brilliant Blue, DAPI, IPTG, kanamycin, Aprotinin, pepstatin, PMSF, β-Glycerophosphate, Sodium Fluoride (NaF), Biotin, and DMSO (Sigma), Luciferase assay kit (Promega #1500), Gateway cloning kit, DMEM, FBS, RPMI, Opti-MEM medium, Met^-/Cys^- DMEM, dialyzed FBS, trypsin-EDTA, L-glutamine, PBS, Lipofectamine 2000, Oligofectamine, (Invitrogen), PEI (Polysciences), milk powder (Warana), protein G agarose beads, Streptavidin sepharose beads, Glutathione sepharose beads, MBP beads (GE Healthcare), S-protein beads (Novagen/Calbiochem), HA beads (Covance), LB media (Himedia).

2.1.2 Antibodies

All the antibodies used in the present study are mentioned in the table 1.

Table 1: Antibodies used in the study

Antibody	Dilution WB/IP	Purchased from
Rabbit anti-WWP2	1:2000/4μg per mg cell lysate	Bethyl laboratories
Rabbit anti-PPM1G	1:10000	Bethyl laboratories

Rabbit anti-HA	1:10000/4µg per mg cell lysate	Bethyl laboratories
Mouse anti-WWP1	1:50	Abnova
Mouse anti-FLAG	1:10000	Sigma
Mouse anti-tubulin	1:10000	Sigma
Mouse anti-Actin	1:10000	Sigma
Mouse anti-GST	1:1000	Santa Cruz Biotechnologies
Mouse anti-Myc 9E10	1:1000	Santa Cruz Biotechnologies
Mouse anti-Ubiquitin	1:2000	Millipore
Mouse anti-MBP	1:10000	New England Biolabs
Rabbit anti-DVL2	1:10000/4µg per mg cell lysate	Bethyl laboratories
Rabbit anti-β-catenin	1:10000	Bethyl laboratories
HRP-conjugated anti-mouse and anti-rabbit secondary antibodies	1:10000	Jackson ImmunoResearch
Goat anti-mouse and goat anti-rabbit Rhodamine	1:200	Jackson ImmunoResearch
Goat anti-mouse and Goat anti-rabbit FITC	1:200	Jackson ImmunoResearch

2.1.3 Expression plasmids

TAp73α and ΔNp73α were kindly gifted by Alex Zaika, Vanderbilt University. Full-length p73 and ΔNp73 were cloned into Myc and HA mammalian destination vectors

using gateway cloning method (Invitrogen). P73 domain deletions were cloned in SFB destination vector. WWP2, WWP1, HACE1, E6AP, and PPM1G were cloned into SFB (S-protein/Flag/streptavidin binding protein (SBP) triple tag), GFP, and Myc mammalian destination vectors using the Gateway cloning technology (Invitrogen). WWP2 domain deletions were cloned into Myc-destination vector. WWP1 domain deletions were cloned into SFB-destination vector. PPM1G domain deletions were cloned into SFB mammalian destination vector using Gateway cloning. Bacterially expressing GST-p73, GST-ΔNp73, GST-PPM1G, MBP-WWP1, MBP-WWP2, GST-WWP2, GST-WWP1 and GST-HACE1 were generated by using gateway technology. Ubiquitin WT and all the mutants were cloned into hemagglutinin (HA) mammalian destination vector. Flag-tagged Dvl2 was purchased from Addgene. Dvl2 domain deletions were cloned into SFB-destination vectors. All the plasmid constructs generated in the present study are mentioned in table 2.

Table 2: Plasmids used in the study

Plasmid Name	Descrption	Reference
Flag-TAp73α	Ampicillin resistance	A Kind gift by Alex Zaika, Vanderbilt University
Donor-TAp73α	Kanamycin resistance	Present study
Myc-TAp73α	Ampicillin resistance	Present study
HA-TAp73α	Ampicillin resistance	Present study
GST-TAp73α	Ampicillin resistance	Present study
MBP-TAp73α	Ampicillin resistance	Present study
Flag-ΔNp73α	Ampicillin resistance	A Kind gift by Alex

		Zaika, Vanderbilt University
Donor-ΔNp73α	Kanamycin resistance	Present study
Myc-ΔNp73α	Ampicillin resistance	Present study
HA-ΔNp73α	Ampicillin resistance	Present study
GST-ΔNp73α	Ampicillin resistance	Present study
MBP-ΔNp73α	Ampicillin resistance	Present study
Donor-p73 D1	Kanamycin resistance	Present study
Donor-p73 D2	Kanamycin resistance	Present study
Donor-p73 D3	Kanamycin resistance	Present study
Donor-p73 D4	Kanamycin resistance	Present study
Donor-p73 D5	Kanamycin resistance	Present study
Donor-p73 D6	Kanamycin resistance	Present study
Donor-p73 ΔOD	Kanamycin resistance	Present study
SFB-p73 D1	Ampicillin resistance	Present study
SFB-p73 D2	Ampicillin resistance	Present study
SFB-p73 D3	Ampicillin resistance	Present study
SFB-p73 D4	Ampicillin resistance	Present study
SFB-p73 D5	Ampicillin resistance	Present study
SFB-p73 D6	Ampicillin resistance	Present study
SFB-p73 ΔOD	Ampicillin resistance	Present study
Donor-WWP2 WT	Kanamycin resistance	Present study
SFB-WWP2 WT	Ampicillin resistance	Present study
Myc-WWP2 WT	Ampicillin resistance	Present study

GFP-WWP2 WT	Ampicillin resistance	Present study
GST-WWP2 WT	Ampicillin resistance	Present study
MBP-WWP2 WT	Ampicillin resistance	Present study
Donor-WWP2 CA	Kanamycin resistance	Present study
SFB-WWP2 CA	Ampicillin resistance	Present study
Myc-WWP2 CA	Ampicillin resistance	Present study
GFP-WWP2 CA	Ampicillin resistance	Present study
GST-WWP2 CA	Ampicillin resistance	Present study
MBP-WWP2 CA	Ampicillin resistance	Present study
Donor-WWP1 WT	Kanamycin resistance	Present study
SFB-WWP1 WT	Ampicillin resistance	Present study
Myc-WWP1 WT	Ampicillin resistance	Present study
GFP-WWP1 WT	Ampicillin resistance	Present study
GST-WWP1 WT	Ampicillin resistance	Present study
MBP-WWP1 WT	Ampicillin resistance	Present study
Donor-WWP1 CS	Kanamycin resistance	Present study
SFB-WWP1 CS	Ampicillin resistance	Present study
Myc-WWP1 CS	Ampicillin resistance	Present study
GFP-WWP1 CS	Ampicillin resistance	Present study
GST-WWP1 CS	Ampicillin resistance	Present study
MBP-WWP1 CS	Ampicillin resistance	Present study
SFB-HACE1 WT	Ampicillin resistance	Present study
Myc-HACE1 WT	Ampicillin resistance	Present study
GFP-HACE1 WT	Ampicillin resistance	Present study

GST-HACE1 WT	Ampicillin resistance	Present study
MBP-HACE1 WT	Ampicillin resistance	Present study
SFB-HACE1 CS	Ampicillin resistance	Present study
Myc-HACE1 CS	Ampicillin resistance	Present study
GFP-HACE1 CS	Ampicillin resistance	Present study
GST-HACE1 CS	Ampicillin resistance	Present study
MBP-HACE1 CS	Ampicillin resistance	Present study
Donor-WWP1 D1	Kanamycin resistance	Present study
Donor-WWP1 D2	Kanamycin resistance	Present study
SFB-WWP1 D1	Ampicillin resistance	Present study
SFB-WWP1 D2	Ampicillin resistance	Present study
Donor-WWP2 D1	Kanamycin resistance	Present study
Donor-WWP2 D2	Kanamycin resistance	Present study
Donor-WWP2 D3	Kanamycin resistance	Present study
Donor-WWP2 D4	Kanamycin resistance	Present study
Donor-WWP2 D5	Kanamycin resistance	Present study
Myc-WWP2 D1	Ampicillin resistance	Present study
Myc -WWP2 D2	Ampicillin resistance	Present study
Myc -WWP2 D3	Ampicillin resistance	Present study
Myc -WWP2 D4	Ampicillin resistance	Present study
Myc -WWP2 D5	Ampicillin resistance	Present study
Donor-UbWT	Kanamycin resistance	Present study
Donor-UbK48WT	Kanamycin resistance	Present study
Donor-UbK48R	Kanamycin resistance	Present study

HA-UbWT	Ampicillin resistance	Present study
HA-UbK48WT	Ampicillin resistance	Present study
HA-UbK48WT	Ampicillin resistance	Present study
Donor-PPM1G	Kanamycin resistance	Present study
SFB-PPM1G	Ampicillin resistance	Present study
Myc-PPM1G	Ampicillin resistance	Present study
GFP-PPM1G	Ampicillin resistance	Present study
GST-PPM1G	Ampicillin resistance	Present study
MBP-PPM1G	Ampicillin resistance	Present study
Donor-PPM1G D441A	Kanamycin resistance	Present study
Donor-PPM1G D491A	Kanamycin resistance	Present study
Donor-PPM1G D496A	Kanamycin resistance	Present study
Donor-PPM1G D3A3	Kanamycin resistance	Present study
SFB-PPM1G D441A	Ampicillin resistance	Present study
SFB-PPM1G D491A	Ampicillin resistance	Present study
SFB-PPM1G D496A	Ampicillin resistance	Present study
SFB-PPM1G D3A3	Ampicillin resistance	Present study
GFP-PPM1G D441A	Ampicillin resistance	Present study
GFP-PPM1G D491A	Ampicillin resistance	Present study
GFP-PPM1G D496A	Ampicillin resistance	Present study
GFP-PPM1G D3A3	Ampicillin resistance	Present study
GFP-PPM1G D441A	Ampicillin resistance	Present study
GST-PPM1G D3A3	Ampicillin resistance	Present study
Donor-PPM1G D1	Kanamycin resistance	Present study

Donor-PPM1G D2	Kanamycin resistance	Present study
Donor-PPM1G D3	Kanamycin resistance	Present study
Donor-PPM1G D4	Kanamycin resistance	Present study
Donor-PPM1G AD	Kanamycin resistance	Present study
Donor-PPM1G ΔAD	Kanamycin resistance	Present study
SFB-PPM1G D1	Ampicillin resistance	Present study
SFB-PPM1G D2	Ampicillin resistance	Present study
SFB-PPM1G D3	Ampicillin resistance	Present study
SFB-PPM1G D4	Ampicillin resistance	Present study
SFB-PPM1G AD	Ampicillin resistance	Present study
SFB-PPM1G ΔAD	Ampicillin resistance	Present study
Flag-Dvl2	Ampicillin resistance	Addgene
Donor-Dvl2	Kanamycin resistance	Present study
Myc-Dvl2	Ampicillin resistance	Present study
SFB-Dvl2	Ampicillin resistance	Present study
GST-Dvl2	Ampicillin resistance	Present study
TOP-Flash	Ampicillin resistance	Addgene
FOP-Flash	Ampicillin resistance	Addgene
Wnt3a	Ampicillin resistance	A kind gift by Dr. Lekha Dinesh Kumar
Wnt5a	Ampicillin resistance	A kind gift by Dr. Lekha Dinesh Kumar
Donor-UbK63WT	Kanamycin resistance	Present study
Donor-UbK63R	Kanamycin resistance	Present study

HA-UbK63WT	Ampicillin resistance	Present study
HA-UbK63R	Ampicillin resistance	Present study
Donor-UbK6R	Kanamycin resistance	Present study
Donor-UbK11R	Kanamycin resistance	Present study
Donor-UbK27R	Kanamycin resistance	Present study
Donor-UbK29R	Kanamycin resistance	Present study
Donor-UbK33R	Kanamycin resistance	Present study
Donor-UbK6WT	Kanamycin resistance	Present study
Donor-UbK11WT	Kanamycin resistance	Present study
Donor-UbK27WT	Kanamycin resistance	Present study
Donor-UbK29WT	Kanamycin resistance	Present study
Donor-UbK33WT	Kanamycin resistance	Present study
HA-UbK6R	Ampicillin resistance	Present study
HA -UbK11R	Ampicillin resistance	Present study
HA-UbK27R	Ampicillin resistance	Present study
HA-UbK29R	Ampicillin resistance	Present study
HA-UbK33R	Ampicillin resistance	Present study
HA-UbK6WT	Ampicillin resistance	Present study
HA-UbK11WT	Ampicillin resistance	Present study
HA-UbK27WT	Ampicillin resistance	Present study
HA -UbK29WT	Ampicillin resistance	Present study
HA-UbK33WT	Ampicillin resistance	Present study
Donor-UbK0	Kanamycin resistance	Present study
HA-UbK0	Ampicillin resistance	Present study

All the primers (sequences) used for cloning the above-mentioned genes are provided in Appendix I.

2.1.4 SiRNA and shRNA

SiRNA

WWP2 siRNA described earlier [216] and prevalidated siRNAs for PPM1G (catalog numbers S102658684 and S102658691) were purchased from Qiagen.

ShRNA

WWP2 shRNA (shRNA1, 5=-CAGGAUGGGAGAUGAAAUAUU-3=; shRNA2, 5= ACAUGGAGAUACUGGGCAAUU-3=)

WWP1 shRNA (shRNA1, 5=-ATTGCTTATGAACGCGGCT-3=; shRNA 2, ACAACACACCTTCATCTCC-3=)

Both WWP2 and WWP1 shRNA were purchased from Open Biosystem.

2.1.5 Cell lines

HeLa cells, HEK293T, and BOSC23 cell lines were used in the present study wherever indicated. All the cells were cultured and maintained in RPMI 1640 supplemented with 10% serum and 1% antibiotic (penicillin-streptomycin) at 37° C with 5% CO_2.

2.2 Buffers and media

The buffers and media used in the present study is mentioned in the table 3.

Table 3: Buffers and media used in the study

Buffers	**Composition**
20x TBST (Per 1L)	121.4g Tris 175g NaCl 40ml Tween 20 Adjust to pH 8.0 Make upto 1L with ddH_2O

1x NETN (Per 1L)	5ml NP-40 20ml 1M Tris-HCl, pH8.0 20ml 5M NaCl 2ml 0.5M EDTA Make upto 1L with ddH_2O
10x PBS (Per 1L)	80g NaCl 2g KCl 11.50g $Na_2HPO_47H_2O$ 2g KH_2PO_4 Make upto 1L with ddH_2O
Sodium Citrate Buffer (Per 100ml)	1.12g Sodium Citrate Make up to 100mL with ddH_2O
Transfer Buffer (Per 1L)	14.4g Glycine 30.725g Tris 1.875g 20% SDS solution 150ml Methanol Make upto 1L with ddH_2O
10x TGS (Per 1L)	30.26g Tris base 144.13g Glycine 10.0g SDS Make upto 1L with ddH_2O
Milk blocking buffer (Per 1L)	50g Milk powder Make upto 1L with TBST
SSTE Buffer (Per 1L)	1mM EDTA, pH8.0 (2ml of 0.5M EDTA) 120mM NaCl (24ml of 5M Nacl) 50mM Tris pH8.0 (50ml of 1M) 1.5 %(15g) Sarcosyline Make upto 1L with ddH_2O
Hypotonic Propidium Iodide Lysis Buffer	1% Sodium Citrate 0.1% Triton X-100 200 Units/mL RNase A 40µg/mL Propidium Iodide
0.5% Triton buffer	20mM HEPES pH 7.4 50mM NaCl 3mM Sucrose 0.5% Triton X100
2X Lamelli-SDS sample buffer	100mM Tris pH 6.8 20% glycerol 4% SDS 0.02gms Bromophenol Blue 10% β Mercaptoethanol

Coomassie staining solution	10% Acetic acid 10% Isopropanal 0.25% Coomassie Brilliant Blue R250
Coomassie destaining solution	10% acetic acid 10% Glycerol
GST elution buffer	10mM reduced glutathione 50mM Tris-HCL pH 8.0
MBP elution buffer	20mM Tris pH 7.4 200mM NaCl 1mM EDTA 10mM Maltose
TAE buffer	Tris-acetate 40 mM EDTA (pH 8.0) 2 mM
SDS running buffer (Per 1L)	Tris-base 30.2 gm Glycine 144 gm SDS 10 gm Water to 1000 ml
SDS PAGE stacking gel (5%) for 5ml	Distilled water 3.4 ml 0.5M Tris (pH 6.8) 0.625 ml 30% Acrylamide mix 0.85 ml 10% SDS 0.05 ml 10% APS 0.05 ml TEMED 5μL
SDS PAGE resolving gel (10%) for 20ml	Distilled water 7.9 ml 30% Acrylamide mix 6.6 ml 1.5M Tris (pH 8.8) 5 ml 10% SDS 0.2 ml 10% APS 0.2 ml TEMED 0.008 ml
In vitro ubiquitination buffer	40mM Tris-HCl (pH 7.6) 2mM DTT 5mM MgCl2 0.1M NaCl 2mM ATP
Denaturing lysis buffer	50mM Tris-HCL(pH 7.5)-500ul of 1M 100mM β-mercaptoethanol- 7ul of 14.4M 1% SDS-1ml of 10% 5mM EDTA-100ul of 0.5M Make up 10 ml with milliQ Boil for 10 min Add protease inhibitors

Cell culture medium	RPMI 1640 (500ml) DBS (50ml) Penstrep (5.5ml)
Luria Broth medium (Per 1L)	10g Tryptone 5g Yeast Extract 5g NaCl (Add 15g agar for making LB plates)

2.3 Methods

2.3.1 Polymerase chain reaction (PCR)

PCR amplification of the gene of interests was carried out by following the method mentioned in table 4.

Table 4: PCR methodology

Materials required	10x PCR Reaction Buffer Taq DNA polymerase Template DNA 10mM dNTPs Forward Primer Reverse Primer DMSO Water Thermal Cycler		
Reaction Mixture	10X PCR Buffer -5µL dNTPs -2µL Template DNA -1-2µL Forward Primer -2.5 µL Reverse Primer -2.5µL Taq Polymerase -0.5 to 0.8µL DMSO -1.5µL Water -x Total Reaction Volume -50µL		
Cycling conditions			
Step	Temperature	Time	No. of Cycles

Initial Denaturation	95°C	5 min	1
Denaturation	95°C	1 min	30
Annealing	55°C	1 min	
Extension	72°C	1 min/Kb	
Final Extension	72°C	7 min	1
Final Temperature	4°C	Forever	

Amplified PCR products were run on Agarose gel to check for the amplification of gene of interests.

2.3.2 Agarose gel electrophoresis

PCR products or plasmids were analyzed by agarose gel electrophoresis. The samples were mixed with 6X loading dye (0.25% bromophenol blue and 0.25% xylene cyanol and 30% glycerol in water) and loaded onto a pre-cast gel, the percentage of gel ranged from 0.7 to 3 %, depending on the size of the DNA sample. Ethidium bromide at 1 μg/ml was included in the gel. The gel was visualized by fluorescence under UV-light.

2.3.3 Site-directed mutagenesis

The various mutant plasmids were generated by using PCR-based site-directed mutagenesis protocol (Stratagene). Briefly, primers carrying the desired nucleotide changes were used in a PCR reaction to amplify the nascent mutant plasmids from the wild type parent plasmid. The PCR reaction was set up according to manufacturer's protocol (Table 5) using a high-fidelity Pfu DNA polymerase and donor-plasmids of the desired gene as the template. Following reaction mixture and the cycling conditions were used for site-directed mutagenesis.

Table 5: Site-directed mutagenesis methodology

Reaction Mixture	10X PCR Buffer -5µL dNTPs -1µl dNTPs Template DNA -5-50ng Forward Primer -125ng Reverse Primer -125ng Pfu Polymerase -1µl (10mM) Water (Sigma) - x Total Reaction Volume -50µL	
Cycling conditions		
Temperature	Time	No. of Cycles
95°C	30 sec.	1
95°C	30 sec.	12 (Point mutation) or 16 (single aa change) or 18 (multiple aa change)
55°C	1 min	
68°C	1 min/Kb	
4°C	Forever	

PCR product was incubated with 1ul of Dpnl restriction enzyme for 2-3 hours at 37ºC, following which Dpn1 treated PCR product was transformed into DH5α competent bacterial cells. Mutant colonies were screened and confirmed using DNA sequencing.

2.3.4 Gateway cloning

Gateway cloning is the highly efficient gene cloning technology. It comprises two primary steps of cloning; the BP reaction and the LR reaction. The PCR products of gene of interest were cloned into the Gateway donor vector by BP reaction (Table 6).

Table 6: BP reaction mixture

BP Reaction	BP Rxn Buffer -2uL pDonr Vector -2uL

	PCR Product -2uL BP clonase -2uL Water -2uL Total Reaction Volume -10uL

BP reaction mix was incubated at room temperature for 1hr, followed by proteinase K treatment (2μL) at 37°C for 10 min. 5uL of the reaction mix was transformed into DH5α competent cells (Transformation into DH5α competent cells; plasmid constructs added into DH5α competent cells and incubated on ice for 30 min. followed by heat shock at 42°C for 1 min. 800μL LB broth was added and transformed DH5α cells were incubated at 37°C in shaking incubator at 220 rpm for 1hr.; after 1 hr. cells were centrifuged at 6000 rpm for 1 min.; the supernatant was discarded, and the pellet was resuspended in 100μL of LB broth). Bacterial cells were spread on LB agar plates containing antibiotic kanamycin (30ug/ml). Plates were incubated at 37°C for overnight. The bacterial colonies were inoculated into 5mL LB broth containing 5uL kanamycin and incubated overnight in shaking incubator at 220 rpm. Next day, Plasmids were prepared using Plasmid miniprep kit (QIAprep miniprep). The donor plasmids generated by BP reaction were given for plasmid-DNA sequencing to confirm the positive clones.

The donor plasmids obtained by BP reaction were cloned into the Gateway destination vectors by LR reaction (Table 7).

Table 7: LR reaction mixture

LR Reaction	LR Rxn Buffer -2uL Entry clone (BP product) -2uL pDest Vector -2uL LR clonase -2uL Water -2uL Total Reaction Volume -10uL

The LR reaction mixture was incubated at room temperature for 1hr, followed by proteinase K treatment (2μL) at 37°C for 10 min. 5uL of the reaction mix was transformed into DH5α competent cells. Bacterial cells were spread on LB agar plates containing antibiotic ampicillin (50ug/ml). Plates were incubated at 37°C for overnight. The bacterial colonies were inoculated into 5mL LB broth containing 5uL ampicillin and incubated overnight in shaking incubator at 220 rpm. Next day, Plasmids were prepared using Plasmid miniprep kit (QIAprep miniprep). The destination plasmids obtained by LR reaction were given for plasmid-DNA sequencing to confirm the positive clones. The positive clones were amplified through DH5α transformation and plasmid DNA maxiprep (Invitrogen). The expression plasmids generated through LR were used for studies in the mammalian and the bacterial cells.

2.3.5 Plasmid Transfection of mammalian cells

293T cells or HeLa cells were transfected with various plasmids as per the designed experiments using Lipofectamine 2000 (Invitrogen) reagent or PEI.

2.3.5.1 Plasmid transfection using Lipofectamine 2000

For transfection with Lipofectamine, cells were plated in antibiotic-free medium 24 h before transfection and were transfected at a confluency of 70-80% as per the manufacturer's protocol. The plasmid of interest was incubated in serum free media, and Lipofectamine was incubated in serum free media for separately 5 minutes. The plasmid and the Lipofectamine mixtures (Table 8) were mixed gently and incubated at room temperature for 20 min.; the transfection mixture was added dropwise to the cells. Transfection media was replaced with the fresh complete medium after 6 hrs. of transfection and cell are harvested after 24 hours.

Table 8: Lipofectamine plasmid-transfection methodology

Culture vessel	Surface area per well	Volume of plating medium	Volume of dilution medium	DNA	Lipofect-amine 2000
96 well	$0.3cm^2$	100µl	2x25µl	0.2µg	0.5µl
24 well	$2cm^2$	500µl	2x50µl	0.8µg	2µl
12 well	$4cm^2$	1ml	2x100µl	1.6µg	4µl
6 well	$10cm^2$	2ml	2x250µl	4µg	10µl
60mm	$20cm^2$	5ml	2x500µl	8µg	20µl
10cm	$60cm^2$	15ml	2x1.5ml	24µg	60µl

2.3.5.2 Plasmid transfection using PEI

Cells were plated in the cell culture dishes one day before transfection in RPMI1640 supplemented with FBS and penstrep (complete medium). All the reagents were brought to room temperature before starting transfection. Plasmid-DNA was diluted in serum-free medium and PEI was added (Table 9)

Table 9: PEI plasmid-transfection methodology

Culture dish	Volume of serum free medium (µL)	Plasmid-DNA concentration (µg)	PEI (1mg/ml) concentration (µg)
6-well	200	3	9
10cm	1000	7-8	21

15cm	2000	11-12	33

The plasmid-DNA/PEI mixture was incubated for 15 minutes at room temperature. The mixture was added to cells, and mixed properly by rocking the culture plate back and forth. Cells were incubated at 37°C in a CO_2 incubator. The transfected cells were harvested at 24-48 hours post-transfection

2.3.6 RNA interference

2.3.6.1 SiRNA

Cells were plated in a manner that they were 30-50% confluent on the day of transfection. Cells were washed with serum-free medium, and the serum-free medium was added to the cells as per plate size. SiRNA was diluted in the serum-free medium, and oligofectamine was diluted in serum-free media, separately (Table 10). Both the complexes were incubated at room temperature for 5 min. Diluted siRNA was mixed gently with diluted oligofectamine and incubated at room temperature for 15 min. The final transfection mixture was added dropwise to the cells and mixed properly by gentle rocking. Cells were incubated for 4 hrs., and the growth medium containing 10% FBS was added to the plates without removing the previous medium. Cells were incubated overnight at 37°C in a CO2 incubator. After overnight incubation, the siRNA transfection was repeated using the same protocol. Cells were harvested after 24-48 hours of second round siRNA transfection. The knockdown was detected by checking the protein levels through western blotting. (Note: SiRNA transfection is carried out in antibiotic free medium)

Table 10: SiRNA transfection methodology

Culture	Cell	Oligofec	RPMI	SiRNA	RPMI	Serum	10%

vessels	number plated	-tamine (µl)	(µl)	(µl)	(µl)	free medium	serum medium
96 well	0.15 x 10^5	0.4	2.6	1	16	0.08ml	0.1ml
24 well	0.37 x 10^5	1	6.5	2.5	40	0.2ml	0.25ml
12 well	0.75 x 10^5	2	8	5	85	0.4ml	0.5ml
6 well	1.5 x 10^5	4	11	10	175	0.8ml	2ml
60mm	3 x 10^5	6.4	17.6	16	280	1.28ml	3.5ml
100mm	9 x 10^5	17.2	52.8	48	840	3.84ml	10.5ml

2.3.6.2 ShRNA

Plasmids containing the shRNA of interest were either transfected transiently or were stably transfected. Transient transfection of shRNA was performed using either Lipofectamine 2000 or PEI (as per the method explained before). Stable integration of shRNA was performed by transfecting shRNA along with retroviral packaging vector PCL-Ampho into BOSC23 packaging cells. The supernatant containing the packed viruses (viral medium) was collected at 48 and 72 hours of transfection. The viral medium was then added to the target cells in the presence of polybrene (8µg/mL). Two days later, cells were cultured in medium containing puromycin for the selection of stable clones. The clones stably expressing the desired shRNA were identified and verified through western blotting and immunostaining using specific antibodies. A similar protocol was used to generate stable cell lines that expressed control shRNA.

2.3.7 Western blotting or immunoblotting

At 24 or 48 h of transfection, cell culture media was removed, cells were collected in 1X PBS by scraping them off the plate. Cells were harvested by centrifugation at 4000 RPM for 5 min. at 4° C. Pellet was washed twice with ice-cold 1X PBS and

lysed with ice-cold NETN lysis buffer (20mM Tris-HCl, pH 8.0, 100mM NaCl, 1mM EDTA, 0.5% Nonidet P-40) containing protease inhibitors (0.5 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml pepstatin) for 30 min. on ice, and then centrifuged at 14000 RPM for 10 min. at 4°C. Protein concentration in the supernatant (cell lysate) was estimated by Bradford assay. 20ug of the protein lysate was mixed with 6X SDS loading dye (100 mM Tris pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 200 mM β-mercaptoethanol), boiled for 5 minutes at 99°C. Protein samples were resolved electrophoretically on SDS-polyacrylamide gels and transferred onto PVDF-membrane (Amersham Biosciences) using a semi-dry transfer apparatus (Biorad) for 1 h at a constant current of 500 mA. Membranes were blocked for 30 min. with 5% non-fat dry milk powder dissolved in 1X PBS and then immunoblotted overnight with primary antibody diluted in blocking buffer. Membranes were washed four times (each 5 min.) with 1X TBST followed by incubation with secondary antibody conjugated with horseradish peroxidase (HRP) for one hour at room temperature. The membrane was washed four times with 1X TBST (each wash for 5 min.), and the specific proteins on the membrane were detected by using enhanced chemiluminescent (ECL) reagents in 1:1 ratio (Amersham).

2.3.8 Immunoprecipitation

Protein-A or protein G beads (50 µl of 50% agarose beads) were washed twice with NETN lysis buffer. 2-5 µg of specific antibody was added to beads (in 1ml NETN) and were incubated with beads for 1 h at 4°C on a rotary shaker. Beads were collected by spinning at 500 g for 2 min. and supernatant was removed. 200-600 µg of total protein (mammalian cell lysate or bacterial cell lysate) was added to the beads and incubated for 2 hrs at 4°C on a rotary shaker. Beads were washed four times with the lysis buffer, each time by centrifuging at 500 g for 2 min at 4°C. After discarding the

final supernatant, beads were boiled in the equal volume of 2X SDS loading buffer (Lamelli Buffer) for 5 min. at 95°C. Bound protein complexes were collected by brief centrifugation (1 min.) at 14000 RPM and the supernatant containing the eluted protein fraction was resolved in SDS gel and analyzed by western blotting technique. For denaturing immunoprecipitation, cells were harvested in 1X PBS and pelleted. Cell pellet was resuspended in denaturing lysis buffer (50mM Tris-HCL, 100mM β-mercaptoethanol, 1% SDS, 5mM EDTA, 0.5mM PMSF, 1 mg/ml aprotinin and 1 mg/ml pepstatin) (200μl for 100mm culture dish) and boiled for 10 min at 99°C. Cells suspension was briefly sonicated. Ice-cold 1X NETN (800μl) was added to the suspension after sonication and incubated on ice for 20 min., cells were centrifuged at 14000 rpm for 10 min. The supernatant was collected and used for immunoprecipiation by following the standard protocol.

2.3.9 Tandem affinity purification

HEK293T cells were transfected with S-protein/FLAG/SBP (streptavidin binding protein)-triple tagged WWP2/Dvl2 and then three weeks later puromycin-resistant colonies were selected and screened for WWP2/Dvl2 expression. The positive stable cells were then maintained in RPMI1640 supplemented with 10% FBS and 2 μg/ml puromycin. The stable cells (harvested cells from ~30 tissue culture plates of 10cm size) were collected in 1X PBS by scraping them off the plates, and were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40) containing 50 mM β-Glycerophosphate, 10 mm NaF, 0.5 mM PMSF, 1 μg/ml of each Pepstatin and Aprotinin on ice for 20 minutes. After removal of cell debris by spinning, cell lysates were incubated with streptavidin sepharose beads for 1 hour at 4°C. The bound proteins were washed three times with 1X NETN and then eluted twice with 2 mg/ml Biotin for 60 minutes at 4 °C. The eluates were incubated with S-

protein agarose beads for 1 hour at 4 °c and then washed three times with 1X NETN. The proteins bound to S-protein agarose beads were resolved by SDS-PAGE and visualized by Coomassie staining. Proteins present in the gels were analyzed by Mass Spectroscopy.

2.3.10 GST/MBP recombinant protein purification from bacteria

A bacterial clone expressing the recombinant protein was grown overnight in 5ml of LB medium with 50ug/ml ampicillin and the next day culture was transferred into 500ml of LB medium with 50 ug/ml ampicillin. Bacterial culture was allowed to grow at 37°C until reached an OD600 of 0.6-0.8 and then induced with IPTG (1 mM) for 4 h at 37°C or overnight at 16°C to induce fusion protein expression. Subsequently, bacterial culture was centrifuged at 4000 RPM for 10 minutes (At this point bacterial cell pellets can be stored at -20° C for later use). The bacterial cell pellet was washed once with PBS and resuspended in 10ml of lysis buffer (1X NETN and protease inhibitors) followed by sonication (45 sec. pulse was given three times). The cell lysate was centrifuged for 10 minutes at 14000 RPM. The supernatant containing the GST fusion proteins was added to the 200µl of 50% GST/MBP beads. After 2 hours of incubation at 4°C, beads were washed three times with 1 ml lysis buffer. Purified proteins were eluted by using GST/MBP elution buffer. The recombinant proteins were analyzed by SDS-PAGE followed by either Coomassie staining or western blotting.

2.3.11 Apoptosis

Apoptosis was measured by Nicoletti method (Nicoletti et al., 1991). Cells treated with appropriate apoptotic stimuli for the indicated times were harvested by centrifugation at 800g for 5 minutes at room temperature. Cells were washed once with PBS, and then resuspended in hypotonic PI lysis buffer (1% sodium citrate, 0.1%

Triton X-100, 0.5 mg/ml RNase A, 40 µg/ml propidium iodide). Cell nuclei were then incubated for 30 min. at 30°C and were subsequently analyzed by FACS. The hypodiploid nuclei in the histograms were considered and represented as percentage of apoptotic cells.

2.3.12 ^{35}S met/cys pulse-chase assay

Cells (HEK293T) were transfected with various combinations of plasmids/siRNAs or treated with cisplatin. Cells were washed first with 1X PBS and then with Met^-/Cys^- 1X DMEM supplemented with dialyzed FBS (10%). Cells were then incubated with Met^-/Cys^- DMEM supplemented with dialyzed FBS in the incubator for 1h (met/cys starvation). Cells were taken out from the incubator, and the culture media was removed. Cells were placed behind the radioactivity protective shield and DMEM supplemented with ^{35}S met/cys (200µCi) was added to the cells. Plates containing radioactive media were then put into the acrylic box and incubated for 1h at 37°C in a CO2 incubator. Plates were taken out and kept behind the radioactivity protective shield; the radioactive media was disposed of in the radioactive liquid waste. One set of cells was harvested for 0 time point, other sets of cells were washed twice with 1X PBS and were incubated with normal medium containing 2mM each of cysteine and methionine. Cell plates were put in the acrylic box and incubated at 37°C in a CO2 incubator. Cells were harvested at different time points. Cells were collected in ice-cold PBS and were lysed using the standard cell lysis protocol. Cell lysate were subjected to immunoprecipitation (IP). IP complex is separated on SDS-gel using standard protocol. The gel was transferred onto PVDF membrane, and the membrane was dried. Dried membrane was exposed in a cassette and the signal was detected using phosphorimager. Later the same blots were probed with specific antibodies.

2.3.13 Immunofluorescence

Cells were grown overnight on coverslips and transfected with various combinations of plasmids. Post 24 hrs. of transfection, cells were washed with PBS and then fixed in 3% w/v paraformaldehyde in 1X PBS containing 50 mM sucrose for 15 minutes at room temperature. Cells were permeabilized with permeabilization buffer i.e. 0.5% Triton X-100 buffer containing 20mM HEPES at pH 7.4, 50mM NaCl, 3mM MgCl2 and 300mM sucrose and were incubated for 5 min. at room temperature. Cells were washed twice with 1X PBS and blocked with 3% BSA/PBS for 30 minutes. Cells were incubated with specific primary antibody diluted in the blocking buffer. After 2 hours of incubation, cells were washed thrice with 1X PBS (each wash for 5 minutes). The 1X PBS was removed, and the cells were incubated with specific FITC or Rhodamine-conjugated secondary antibody at 37°C for 30 min. To visualize nuclei, cells were co-stained with DAPI (10 μg/ml). Cells were washed thrice with 1X PBS and after final wash, coverslips containing cell were mounted on the slides using glycerine containing paraphenylenediamine. The cells were analyzed using confocal microscopy facility at CDFD.

2.3.14 Cycloheximide-chase assay

Cells were transfected with various combinations of plasmids and treated with cycloheximide (50ug/ml) 24 hrs. post-transfection. Cells were harvested at different time points, and the protein levels were determined by using the standard protocol for western blotting/immunoblotting.

2.3.15 *In vivo* ubiquitylation assay

HeLa cells were transfected with various combinations of plasmids. At 24 h post-transfection, cells were treated with MG132 (10μM) for 6 h and the whole-cell extracts were prepared by NETN lysis. The cell lysate was subjected to

immunoprecipitation by using substrate specific antibody or pull-down by affinity trapping the substrate tag. The IP/pull-down complexes were analyzed by detecting the ubiquitination of substrate protein by using either substrate specific antibody or ubiquitin antibody through western blotting.

2.3.16 *In vitro* ubiquitylation assay

The reactions were carried out at 30 °C for 15 min in 25µl of ubiquitylation reaction buffer (40mM Tris-HCl at pH 7.6, 2mM DTT, 5mM MgCl2, 0.1M NaCl, 2mM ATP) containing the following components: 100µM ubiquitin, 20nM E1 (UBE1), 100nM UbcH5b (all from Boston Biochem). The bacterially purified MBP-WWP2 and MBP-WWP1 E3 ligases were added to the reaction mixture. The bacterially purified and GST bound GST-protein, GST-p73, and GST-ΔNp73 were used as the substrate in the reaction. After the ubiquitylation reaction, the GST beads were washed five times with 1X NETN buffer and boiled with an equal volume of SDS-PAGE loading buffer. The ubiquitination of the substrates was determined by western blotting with the substrate-specific antibody.

2.3.17 Gel filtration

The WWP2-WWP1 heterodimeric complex formation under normal conditions and upon cisplatin-induced stress conditions was studied by gel exclusion chromatography. HEK293T cells untreated or treated with cisplatin were harvested, and cell lysate was prepared by using the standard protocol. Sephacryl S-200 (GE Healthcare) columns were equilibrated with 1X NETN (without Triton X-100) at a flow rate of 1ml/min. 0.8ml of cell lysate (1mg/ml) was passed through the Sephacryl S-200 column, and different fractions (fraction size; 500µl) were collected using Bio-Rad 2110 fraction collector at the same flow rate. To determine the molecular weight of the fractions, column was calibrated with high molecular weight markers [range

44KDa to 670 KDa; Ovalbumin (44KDa), Conalbumin (75KDa), Aldolase (158KDa), Ferritin (440KDa), Thyroglobulin (669KDa)] purchased from GE Healthcare Life Science. Blue dextran was used for the void volume of the column. The molecular weight of the protein complex fractions was calculated from the plot. The different fractions that were collected as eluates from the column were concentrated, and the presence of WWP2 or WWP1 as a monomer or multimer was identified by western blotting using specific antibodies for WWP2 and WWP1.

2.3.18 Luciferase reporter assay

Luciferase assay was performed using luciferase assay systems (Promega #1500). Cells were transfected with shRNA. Following 24 hrs. of shRNA transfection, cells were transfected separately with FOP-Flash and TOP-Flash vectors in the absence and presence of Wnt3a plasmids. After 24 hrs, media was removed, and the cells were rinsed twice with 1X PBS. After removing the final wash, the cells were incubated with lysis buffer (1X lysis reagent: CCLR; 20μl/well for a 96-well plate, or 400 μl/60mm culture dish, or 900μl/100mm culture dish). Cells were collected in a microcentrifuge tube and were centrifuged at 14000 rpm for 10 minutes. The cell lysate (supernatant) was transferred to a new tube. 20μl of cell lysate was mixed with 100μl of Luciferase Assay Reagent (LAR), and the amount of light produced was measured in luminometer by using a delay time of 2 sec and a read time of 10 sec.