# Chapter 2

# Materials and methods

*There are no secrets to success. It is the result of preparation, hard work learning from failure. ~ General Colin Powell*

## 2.1 Constructs made in the study

### 1. GFP-β-catenin-FL

For generation of full-length GFP-β-catenin, RFP-FL-β-catenin which was available in lab, was digested with BamH1 and the released insert was ligated in pEGFPC-1 at CIP treated BamH1 site; colonies were screened for the orientation by Sac1 which released the expected 1.7 Kilobase (kb) band indicative of right orientation of insert.

### 2. GFP-β-catenin T41A mutant

In order to check the minimal region required for the Nup358 interaction in β-catenin, we generated various deletion mutants of β-catenin. All the deletions are shown in the Fig 2.1. For generation of GFP-β-catenin T41A mutant, C-myc β-catenin T41A mutant (kind gift from Dr. Christiane) was digested with Apa1/Kpn1, the released 2.3 kb insert was ligated at the Apa1 digested pEGFPC-1 site; clone confirmation was done with 3kb release by Nde1 digestion.

### 3. GFP-β-catenin- ΔN (1–151 a.a)

This clone was generated by digesting the GFP-β-catenin T41A mutant with Kpn/Pml1 and end filling in the presence of Klenow and 1 mM dNTP. Vector bone DNA was gel eluted and self-ligated with ligase. Positive clones were confirmed by the digestion with BamH1 and comparison of the release with parental clone.

**4. GFP-β-catenin- ΔC (634 -781 a.a)**

This clone was generated by digesting the GFP-β-catenin T41A mutant with BbvC1/Sma1 and end filling in the presence of Klenow and 1 mM dNTP. DNA was gel eluted and self-ligated with ligase. Positive clones were confirmed by the digestion with BamH1 and comparison of the release with parental clone.

**5. GFP-β-catenin-ΔNC (ARM)**

GFP-β-catenin- ΔC (634-781 a.a) was digested with Kpn/Pml1 and end filling was carried out in the presence of Klenow and 1 mM dNTP. Vector bone DNA was gel eluted and self-ligated with ligase. Positive clones were confirmed by the digestion with BamH1 and comparison of the release with parental clone.

**6. GFP-β-catenin-ΔNC-N (151-423 a.a) (ARM-N)**

This clone was generated by digesting the GFP-β-catenin–ΔNC mutant with EcoR1 and released fragment was ligated at EcoR1 digested and CIP treated site of linearized pEGFPC1 vector; clone confirmation was done with EcoR1.

**7. GFP-β-catenin–ΔNC-C (423-634 a.a) (ARM-C)**

GFP-β-catenin-ΔNC DNA was digested with Sac1/EcoR1 and after end filling in the presence of Klenow and 1 mM dNTP, DNA was gel eluted and self-ligated with ligase. Positive clones were confirmed by the digestion with BamH1 and comparison of the release with parental clone.

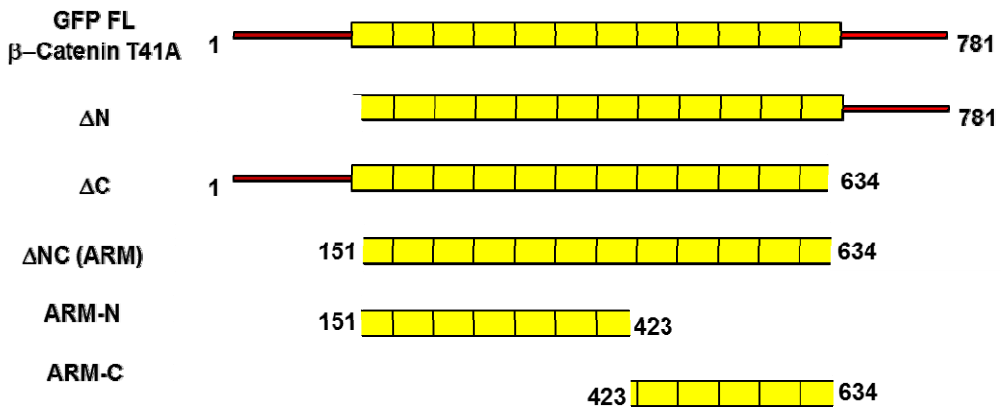


***Fig 2.1 Deletion mutants of β–catenin****. Deletion mutants of β-catenin in pEGFP-C-1-Fl-β-catenin T41A (1-781 a.a) were made to narrow down the region of interaction in β-catenin for Nup358 interaction. Numbers represents the amino acids retained after deletion.*

**8. pET30a-β-catenin-FL (Antigen for β-catenin antibody)**

Full length β-catenin was released from pEGFP-C1 β-catenin with BamH1 digestion and ligated at the BamH1; site of pET30a. Positive clones were confirmed with induction and western blotting against β-catenin specific antibody.

**9. pET30-b-APC Nde/Sca (1211-1495 a.a) (antigen for APC antibody)**

For generation of pET30b Nde/Sca1 pET30b Nde/Xmn was digested with Sca & BamH1 vector backbone was end filled & self-ligated with ligase clones were screened with Nco/Ecor1 digestion. Further confirmation was done after expressing it in BL21 (DE3) pLysS.

**10. pET30a-GFP-FL (GFP antigen)**

pEGFPC-1 was digested with Nhe1 and end filled and second time digested with BamH1, gel elution of 0.8 kb GFP insert was done and insert was ligated

at EcoRV and BamH1 digested pET30a vector. Clone was confirmed with sequencing and expression of the green florescent protein.

**11. pGEX6P2-BPN (1-993 a.a)**

For generating pGEXBPN (1-993 a.a), pEGFPC-1 BPN(1-993 a.a) was digested with Not1 and after end filled digested with Sal1 0.3 kb insert was eluted with ligation was done at Sma1, Xho1 digested pGEX6P2. Clones were confirmed with expression and digestion with Bgl2.

Other constructs, GFP full length Nup358, GFP-BPN, GFP-BPM and GFP-BPC, pGEX β-catenin used in this study were available in the lab and if obtained from other sources have been duly acknowledged.

**2.2 Animals**

For antibody generation against different proteins such as GFP, β-catenin and APC, NZW rabbits of 6 to 8 month old were obtained from the Experimental Animal Facility of National Centre for Cell Science (NCCS, Pune, India). All procedures involving injections of antigen, serum collection from animals were conducted using the protocol approved by the institutional animal ethics committee of NCCS.

**2.3 Medium and Serum**

DMEM and OPTIMEM (Serum-free medium) was obtained from NCCS repository (NCCS, Pune, India). Media was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), and 10 µg/ml ciprofloxacin (Cipla Ltd., India). FBS was heat-inactivated at 56 $^{o}$C (Degree celsius) for 45 mins (minutes) and then stored in 50 ml falcon tubes at -20 $^{o}$C.

## 2.4 General reagents and plastic wares

Restriction Endonucleases, Calf intestinal phosphatases (CIP), T4 DNA ligase, Klenow, 1kb DNA ladder were purchased from New England Biolab (Beverly, MA). Agarose, dNTPs, BSA (bovine serum albumin), Complete Freund's adjuvant (CFA), Incomplete Freund's adjuvant, Kanamycin, Ampicillin, IPTG (Isopropyl-beta-thio galactopyranoside), NiSO4.6H2O (nickel sulphate hexahydrate), Dimethyl sulphoxide (DMSO), Trizma base, Glycine, Sodium dodecyl sulphate, DTT, L-Glutathione Reduced, Tween 20, Acrylamide, Bis Acrylamide, Imidazole, DNA-binding dye (Hoechst-33342) were purchased from Sigma Aldrich ltd. DNA purification kit, PCR clean up kit, Dual-luciferase reporter assay system were purchased from Promega ( Promega, Madison, WI). NE-PER Nuclear and cytoplasmic extraction reagents kit were obtained from Pierce (Pierce, Thermo scientific). GB004 blotting sheets for western blotting, Amicon column filter were purchased from Millipore. Oligofectamine or lipofectamine for transfection, Protein A Sepharose 4B were purchased from Invitrogen. PEI (poly-ethyleneimine) was purchased from Polybioscience. Kaleidoscope protein Marker, Biorad Protein Assay reagent, Affigel 10 or 15 were purchased from Biorad. Glycerol, Triton-X 100, NP40, EDTA, EGTA, HEPES were purchased from (USB). ECL PLUS Western Development Kit, Glutathione Sepharose 4 fast flow was purchased from GE Healthcare. S35 methionine was obtained from (BRIT, Mumbai, India). Normal horse serum (NHS), Mounting media were purchased from Vector laboratories (Vector Laboratories, CA, USA). All plastic wares such as multi-well cell culture plates, 15 and 50 ml centrifuge tubes, and flasks were obtained from BD Pharmingen (Franklin Lakes, NJ).

## 2.5 Cell culture

SW480, HEK293T, MDCK and HeLa S3 cells were grown in Dulbecco's modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and

10 µg/ml ciprofloxacin antibiotic in a humidified incubator at 37°C under 5% CO2. CHO-K1 was grown in Ham's F12 medium with 10% FBS and antibiotics. All these cell lines were obtained from the cell repository of NCCS.

### 2.6 Antibodies used in our study

We used various primary and secondary antibodies for detection of the proteins either by western blotting or immunofluorescence.

#### 2.6.1 Primary antibodies

For various immunofluorescence studies and immunoprecipitation assays polyclonal rabbit antibody against the GFP, β-catenin and APC protein were raised using either full length or region of proteins as an antigen.
Rabbit polyclonal antibodies against human Nup358 was available in the lab against the region corresponding to 2560-2971 amino acids (a.a), mouse anti-RanGAP1 (Santa Cruz Biotechnology), mouse anti-vinculin (Sigma), mouse anti-FXFG-containing nucleoporins (mAb414; Covance), mouse anti-GFP (B-2, Santa Cruz Biotechnology), mouse anti-GFP (Roche), mouse anti-β-catenin (BD Biosciences/ Transduction lab), mouse anti-α-tubulin (Sigma), mouse anti-LaminA/C (Santa Cruz Biotechnology), mouse anti-vinculin (Sigma), mouse active β-catenin (ABC, clone 8E7 Upstate/Millipore), Rabbit P33/37/41 β-catenin, P41/45 β-catenin, P45 β-catenin, (Cell Signaling), Mouse anti 6XHis (H-3, Santa Cruz Biotechnology). Alexafluor molecules (Invitrogen, Molecular Probes Ltd).

#### 2.6.2 Secondary antibodies

Various Alexa Fluor conjugated and HRP conjugated Secondary antibodies which were used in our studies were purchased from different sources.

Alexa Fluor 488 donkey anti-rabbit IgG, Alexa Fluor 488 donkey anti-mouse IgG, Alexa fluor Donkey anti mouse 594, were purchased from Invitrogen (Molecular probes). Anti-Mouse IgG, HRP, Anti-rabbit IgG, HRP, ECL Anti-Mouse IgG, HRP-Linked Whole Ab (from sheep), were purchased from GE Biosciences.

**2.7 Cell transfection**

For immunofluorescence and immunoprecipitation studies cells were grown on glass cover slips in 24-well plate or in 6-well plate respectively. After 6 to 12 hrs.,Transfections were performed using either using PEI, or Lipofectamine 2000 as transfection reagent, cells were transfected with indicated plasmid DNA, followed by complete change of DMEM medium after 6-12 hrs and cells were allowed to grow for another 12-24 hrs, in complete DMEM containing 10% FBS and 10 μg/ml ciprofloxacin antibiotics. After 36-48 hrs of transfection cells were subjected for immunofluorescence analysis and/or immunoprecipitation and western blotting.

For transfection in 6 well formats, 70 % to 80% confluent cells were used. For transfecting the cells, first 100 μL of OPTIMEM was taken in two eppendorf tubes, in one tube 2 μg of DNA and in other 10μL of PEI or Lipofectamine was added and mixed, After 5 min of incubation at the room temperature both the complexes were mixed and kept for another 20 mins. After 20 mins, Transfection mix was applied on the cells from one side, gently for uniform distribution of transfection complex to the cells; plate was swirled gently and kept back in the incubator. Following the same protocol, for transfection in 24 well format, reagent was scaled down to 50μl of OPTIMEM and 2μl of transfection reagent.

## 2.8 RNA Interference

Cells were grown on glass cover slips for 12-24 hrs. For RNAi, cells were transfected with annealed double-stranded RNA oligonucleotides using Oligofectamine or lipofectamine (Invitrogen) as a transfection reagent at a final concentration of 100 nM.

The target gene sequences used for generating the oligonucleotides were as follows: (Murawala, et al. 2009)

1) Control (non-silencing) siRNA, 5'-AATTCTCCGAACGTGTCACGT-3' (Qiagen)
2) Nup358 siRNA, 5'-AAGGTGAAGATGGATGGAATA-3' (Dharmacon)

After 24 hrs of transfection, cells were allowed to grow in DMEM containing 10% FBS and antibiotics for the indicated times. The cells were processed for IF analysis and/or Western blotting 60-72 hrs later.

## 2.9 Generation of polyclonal antibodies against GFP, APC and β-catenin

For generation of polyclonal antibodies, either full length or region of protein were sub-cloned into compatible pET series vector and the recombinant protein was expressed in *Escherichia coli* BL21 (DE3) pLysS cells (Novagen) and purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany). After purification, 250 µg antigen was emulsified with Freund's complete adjuvant and injected intra-dermally in NZW rabbit. Subsequently booster doses were given by subcutaneous injection at the interval of three weeks with 125 µg antigen emulsified with Freund's incomplete adjuvant. Rabbit polyclonal antibodies were raised against the different proteins and serum was collected 10 days after the booster dose. Specific antibodies were enriched by affinity purification using the affi-gel conjugated antigen column.

For affinity purification, 3-4 mg antigen was dialyzed in 80 mM HEPES (and bound with affigel 10 or 15, depending upon the pI of the antigen. Serum with dilution of 1:1 with 1X TBST, was incubated with column for 4-6 hrs and after 5-6 times washing with 1X TBST bound antibodies were eluted using low pH (2.3) glycine buffer. Antibodies were dialyzed in 1X TBS with 10% glycerol for 12 hrs, further antibodies were concentrated using Amicon column filter with cut off of 10 KDa. After concentrating the antibody quantitative gel analysis was done to check the amount of antibodies, suitable aliquots were made and freezed in -80 ultrafreezer and used for various immunofluorescence and immunoprecipitation studies.

Following the above mentioned standard protocol, we successfully generated specific antibodies against the β-catenin, GFP and APC protein.

**2.10 Immunofluorescence**

For IF analysis, cells were fixed 24-48 hours after transfection using chilled methanol for 10 mins. Cells were further incubated with indicated primary antibody in 2% Normal Horse Serum (NHS) made in 1X TBS for 60 mins. Cells were washed with 1X TBS three times and incubated for 60 mins with indicated secondary antibody conjugated to Alexafluor molecules. DNA-binding dye Hoechst-33342, which stains the nucleus, was added to secondary antibody dilution. After secondary binding, the cells were washed thrice with TBS and the dried coverslips were mounted onto slides using mounting media. Coverslips were sealed using nail paint to prevent dehydration and observed under microscope. Images were acquired with a Zeiss Axiovert 200M using a Plan Apochromat 63XNA1.4 oil immersion objective. Projection images were generated from optical sections 100 nm apart, with a section thickness of 700 nm, using the Axiovision Extended

Focus module. Images were processed further in Adobe Photoshop CS3. To avoid the Spectral Bleed-Through artifacts in colocalization of GFP tagged and RFP tagged proteins, confocal microscopy was also performed.

**2.11 Immunoprecipitation and western Blotting**

For immunoprecipitation, cells were washed with ice-cold 1X TBS once and lysed in ice cold cytoskeleton extraction (CSE) buffer, cells were resuspended in CSE buffer and syringe lysed through 22 gauge needle and 24 gauge needle subsequently. To get the soluble fraction, lysate was spun at 12000 rpm, 30 mins at 4$^{o}$C to remove debris. For input, lysate was mixed with equal volume of 3X PAGE loading dye, boiled at 95$^{o}$C for 4 mins. For immunopreciptation 20 µl Protein A dynabeads or n-Sepharose 4 Protein A beads pre-bound with 5-10 µg indicated antibodies were incubated with the cell lysate at 4°C for 2 hrs on rotospin. The immunoprecipitate was washed 3-4 times with the CSE lysis buffer and one time with 1X TBS, eluted in 3X SDS dye and separated on SDS-polyacrylamide gels. The proteins were transferred to PVDF membrane using semidry transfer apparatus, and western blotting was performed using indicated antibodies.

For western blotting, PVDF membranes were incubated with indicated primary antibodies in 0.5 % BSA for 1.30-2.00 hrs, washed with 1X TBST for three times and incubated with secondary antibodies at 1:10,000 dilution. Further they were washed three times with 1X TBST and blots were developed using ECL plus western detection kit.

**2.12 Luciferase reporter assay**

To measure the transactivation activity of β-catenin in different condition we used a dual luciferase reporter assay system which has a pair of luciferase

reporter constructs 16X TOP-FLASH and 16X FOP-FLASH (Randal Moon, Washington university). TOP-FLASH contains 16 copies of the TCF binding sites (represented in the Fig 2.2), and FOP-FLASH contains mutated TCF binding sites. Cells were transiently transfected with one of these luciferase reporters and Renilla (Upstate Biotechnology, Lake Placid, NY, USA) using PEI or lipofecatmine as transfection reagent. Luciferase activity was measured according to the experimental set up i.e in overexpressed condition after 36 hrs or in RNAi condition after 72 hrs of transfection, with the Dual-luciferase reporter assay system.

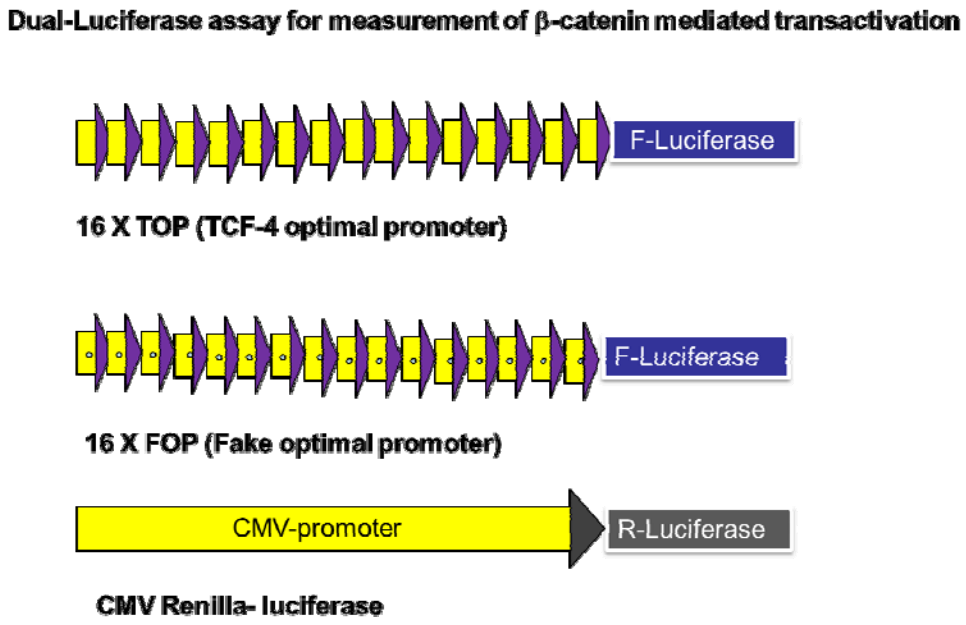


***Fig 2.2 Schematic representation of the* TOP *and* FOP *luciferase reporter construct.*** TOP *and* FOP *luciferase reporters (upper two panels) are widely used for measuring Wnt/β-catenin signaling. The TOP construct contains sixteen binding sites for TCF transcription factors (16×*TCF*) and the firefly luciferase reporter (*F-luc*) under control of a TCF-4 minimal promoter. The FOP construct is identical to the TOP construct, with the exception that the sixteen TCF binding sites are mutated and therefore inactive. The lower most construct contains the renilla luciferase under the control of constitutive active CMV promoter. Renilla serves as transfection control for normalization.*

In transient transfection experiment, SW480 cells were co-transfected with GFP (control) or GFP tagged proteins (GFP-Fl-Nup358 or GFP-BPN, GFP-BPM, and GFP-BPC) along with reporter plasmid TOP or FOP (100 ng) and transfection control renilla (5 ng).

In RNAi experiments in Hela S3 cells, 100nM of Nup358 RNAi or control RNAi were transfected after 24 hrs. Reporter constructs were transfected, after next 24 hrs, 20 mM LiCl was used for Wnt induction. After 24 hrs of LiCl treatment cells were washed with 1X PBS and lysed in 1X PLB and readings were taken in luminometer (Promega Turner Biosystem) from 25 µL of cell lysate.

### 2.13 Large scale purification of BPN in CHO cells from pSecTag2 vector

Since the bacterially expressed BPN was found to be insoluble and heat labile, we made an attempt to get the soluble protein from the eukaryotic secretory vector. We cloned the BPN in pSecTag2 vector according to the mentioned strategy in construct made.

After confirming the His, Myc, BPN positivity of pSecTag2BPN clone in CHO cells by immunofluorescence, we went ahead with large scale transfection and purification of protein from extracellular medium. Here, BPN pSecTag2 constructs containing BPN and pSecTag2 empty vector were transiently transfected into CHO-K1 cells using PEI, as a transfection reagent in 100 mM petri dishes. In serum-free medium (OPTIMEM) and 6-8 h post-transfection; media was changed with fresh OPTI-MEM for better expression of the soluble secretory proteins. The culture supernatant containing the expressed proteins was collected after 72 h and concentrated 20X, using Amicon column. To check the presence of secreted protein in the collected and concentrated supernatant, Western blotting was performed, the column concentrated proteins were subjected to electrophoresis on a 10% SDS-PAGE.

Thereafter, proteins were transferred onto a PVDF membrane using transfer apparatus. Probing of the blot was performed using, specific Mouse anti 6XHis antibody.

**2.14 Nuclear and cytoplasmic fractionation**

HeLa S3 cells were treated with Nup358 siRNA, after 72 hrs. of siRNA transfection cells were fractionated into the cytoplasmic and nuclear fraction using the NE-PER (Nuclear and cytoplasmic Extraction Reagents kit).

After fractionation equal quantity of both nuclear and cytoplasmic fractions was boiled in 3X SDS loading dye and loaded on the 10% gel and western blotting was performed. For checking the purity of nuclear and cytoplasmic fractions laminA/C and tubulin antibody were used respectively. To check the separation of β-catenin in nuclear and cytoplasmic fractions in control and Nup358 RNAi condition mouse β-catenin antibody was used.

**2.15 Stripping of Ni-NTA agarose beads**

To check the specific interaction of β-catenin with nickel, Ni-NTA agarose beads were taken and incubated with 100 mM EDTA for 2 hrs on rotospin, after 2 hrs, beads were washed with 1X TBS twice and used as a negative control for the Ni-NTA pull down either for endogenous proteins or in in-vitro interaction studies with purified proteins.

**2.16 Ni-NTA pull down**

Cells grown in 6 well plates were resuspended and washed with ice-cold TBS twice and pelleted. Cell pellet was resuspended in mild lysis buffer NP40 buffer and syringe lysis was done by passing the cells through 22 gauge needle and 24 gauge needles subsequently. Lysate was spun at 12000 rpm, 30

mins at 4°C to remove cell debris. For input, lysate was mixed with equal volume of 3X PAGE loading dye, boiled at 95°C for 5 mins. For pull down, 20 µL Ni-NTA agarose beads and NTA beads were incubated with the cell lysate at 4°C for 2 hrs on rotospin. The bound complex was washed three times with the 1X TBST buffer and, eluted in 250 mM imidazole, separated on SDS-polyacrylamide gels. The proteins were transferred to PVDF membrane using semidry transfer apparatus, and western blotting was performed using indicated antibodies.

**2.17 Luciferase assay in SW480 cells under nickel induced condition**

In SW480 cells Wnt/β-catenin signaling was assessed under NiSO4 induced condition using the TOP/FOP dual luciferase assay system. 25,000 cells per well grown for 12 h in 24-well plates were transiently transfected with TOP (100 ng) or FOP (100 ng) using PEI as a transfection reagent. Renilla (5 ng) was transfected in each well as an internal transfection control. 24 hrs post transfection cells were treated with various concentration of NiSO4 such as 25 µM, 50 µM, 100 µM, 250 µM, 500 µM. After 24 hrs post induction cells were washed with 1XPBS and lysed in 200 µL of 1X Passive lysis buffer (PLB) and readings were taken in luminometer from 25 µL of lysate following Promega dual luciferase kit protocol.

**2.18 Immunofluorescence studies in SW80 cells for β-catenin under nickel induced condition**

For studying the effect of nickel on subcellular localization of endogenous β-catenin, by immunofluorescence, SW480 cells were plated in 24 well plates with coverslip after 10 to 12 hrs cells were treated with 500 µM of NiSO4. For immunofluorescence, above mentioned protocol was followed.

**2.19 Expression and purification of recombinant proteins**

Expression of different recombinant proteins was done as described below. pET or pGEX transformed colonies of Escherichia coli BL21 (DE3) pLysS harboring the plasmid, were inoculated into 500 ml of LB-kanamycin (50 µg/ml final concentration) or LB-ampicilin (100 µg/ml final concentration) in media and grown at $37^0$C orbital shaker till the optical density reached 0.5 to 0.6 at $A_{600.}$ Induction of protein was done by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside, and the induced culture was further grown for 6 hrs, meanwhile induction profile was checked at a small scale after 4 hrs of IPTG addition. Once induction was confirmed, cells were then harvested by centrifugation at 12,000 rpm at $4^0$C. The harvested cell pellet was dissolved in 25 ml Ni-NTA lysis buffer or Glutathione purification buffer and the cell suspension was sonicated in sonicator (vibra cell sonicator) with 10 cycles of 15 pulses of 5 seconds each, further the lysate was centrifuged at 12,000 rpm for 30 mins at $4^0$C. The soluble fraction of protein was collected and incubated with pre-washed beads of either Ni-NTA beads or glutathione beads for 1 hrs on rotospin. After binding the column was washed 4-5 times with washing buffer containing 20 mM imidazole in case of Ni-NTA or 1X TBS in glutathione purification, and the bound protein was eluted with respective elution buffer. Further eluates were dialyzed against the 1X TBS, pH 7.4 at 4 $^0$C.

At the end, proteins were concentrated, and the purity and quantity was checked on the coomassie stained gels. Suitable aliquots were made and used as and when required for different experiments.

**2.20 Surface plasmon resonance (SPR) measurements**

The kinetics of binding of soluble β-catenin to nickel was determined on the SPR-based biosensor BIACORE 2000 (Bicore AB, Uppsala, Sweden). Binding

analysis was performed in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 50 mM EDTA, 0.05% surfactant P20, pH 7.4) at $25^0$C. Here, a NTA chip was coated with nickel (50RUs) using 500 μM NiSO4. Binding of β-catenin to the immobilized nickel was measured by flowing various concentration of β-catenin at 10 μL/min for 120 seconds, followed by dissociation for 180 seconds. The sensor chip was regenerated with 30 sec pulses of 0.2 M sodium bicarbonate, pH 9.5 and regeneration buffer (10 mM HEPES, 150 mM NaCl, 0.35M EDTA, 0.005%, surfactant P-20 pH 7.4), the specific binding response for β-catenin and GST-β-catenin to nickel was obtained by subtracting the response generated by the control flow cell (Fc-1) from the data obtained from the flow cell (Fc-2) immobilized with nickel. SPR data obtained were evaluated using 1:1 Langmuir binding model with drifting baseline (BIAevaluation 4.1).

**2.21 GST-pull down**

For GST and GST-β-catenin pull down, HEK cell lysate was made in cytoskeletal buffer as mentioned in immunoprecipitation protocol. Soluble cell lysate was incubated for 2 hrs on continuous mixing on rotospin, with 5 μg of GST (negative control) or GST-β-catenin, pre-immobilzed with 20 μl of glutathione beads. After 2 hrs, of binding, beads were washed with 1X TBST, 3 times. Complex was eluted in 50 μl of 3X SDS loading dye and loaded on the 6% gel and western blotting was performed with Nup358 and RanGAP specific antibodies.

**2.22 Composition of buffers and solutions**

1) **1X PBS**

   137 mM NaCl

   2.7 mM KCl

   10 mM $Na_2HPO_4$

   2 mM $KH_2PO_4$

pH was adjusted to 7.4 using HCl

**2) 1X TBS**

20 mM Tris-HCl

137 mM NaCl

pH was adjusted to 7.4 with HCl.

**3) 1X TBST**

0.1% Tween-20 in 1X TBS.

**3) Lysis buffer for Ni-NTA and glutathione purification**

1X TBS

3.5 mM $MgSO_4$

0.5% Triton X-100

0.1% Non-Iodet P-40

1 mM DTT

10 mM PMSF

10 mM EDTA

1 mg/ml Lysozyme

100 µg/ml DNase I

**4) Ni-NTA elution buffer**

1X TBS

250 mM Imidazole

10 mM EDTA

10 mM PMSF

2 mM DTT

**5) GST elution buffer**

1X TBS

20 mM reduced glutathione

10 mM EDTA

10 mM PMSF

2 mM DTT

pH-8.0

**6) Semi dry transfer buffer**

25 mM Tris Base

150 mM Glycine

5% v/v Methanol

pH - 8.9

**7) Cytoskeleton extraction buffer (CSE)**

10 mM Tris-Cl, pH-7.4

100 mM NaCl

1 mM EDTA

1 mM EGTA

1% Triton X-100

10% Glycerol

0.1% SDS

0.5% Na-deoxycholate

1 mM NaF

2 mM $Na_3VO_4$

Mammalian protease Inhibitor cocktail

**8) Low-pH antibody elution buffer**

100 mM Glycine

150 mM NaCl

10% Ethylene Glycol

pH was adjusted to 2.3 using HCl.

**2.23 Nonidet-P40 (NP40) buffer for Ni-NTA pull down**

For Ni-NTA pull down in HEK and SW480 cells for nickel–β-catenin interaction mild lysis buffer was used

150 mM NaCl

1.0% NP-40

50 mM Tris, pH 8.0

**2.24 $NiSO_4$. 6H2O stock soloution**

100 mM solution of NiSO4 (Mol.wt 262.84) was made in autoclaved MilliQ water and stored at room temperature.

**2.25 Statistical analysis**

Dual luciferase assay results are expressed as mean ± standard error of the mean (SEM) for various experimental groups. The statistical differences between groups were analyzed using an unpaired Student's *t* test, and were considered significant where $p < 0.05$.