Chapter 2. Materials and Methods
2.1 Buffers and Solutions:

1) 1x CMF PBS (Calcium and magnesium free PBS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.3 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.25 g</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.04 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0 g</td>
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</tbody>
</table>

All the above components (except NaHCO$_3$ and Glucose) were dissolved in 500mL Milli-Q water, pH adjusted to 7.4 and volume made up to 1 litre. The solution was autoclaved and stored at RT. At the time of use, NaHCO$_3$ and glucose were added, the solution was filtered using a membrane filter with pore size 0.2 µm and stored at 4°C.

2) 1x TBS

20 mM Tris-HCl
137mM NaCl

The above components were dissolved in 500 mL Milli-Q water, pH was adjusted to 7.4 with HCl and volume was made up to 1 litre. It was autoclaved and stored at RT.

3) 1x TBST

0.1% Tween-20 in 1x TBS
4) **Semi Dry Transfer Buffer (for low molecular weight protein transfer < 90 KDa)**

- 25 mM Tris Base
- 150 mM Glycine
- 5% v/v Methanol

pH should be 8.9 without adjustment

**For high molecular weight protein transfer:**

- 50 mM Tris Base
- 38 mM Glycine
- 0.02% SDS
- 5% v/v Methanol

pH should be 8.9 without adjustment

5) **Bacterial lysis buffer for purification of His tagged proteins:**

1x PBS containing;

- 3.5 mM MgSO$_4$
- 0.5% Triton X-100
- 0.1% Non-Iodet P-40
- 10 mM PMSF
- 1 mg/ml Lysozyme
- 100 µg/ml DNase I
6) **Ni-NTA beads elution buffer (pH-7.4)**

1x PBS containing:

- 250 mM Imidazole (pH-8.0)
- 10 mM PMSF

7) **Bacterial lysis buffer for purification of GST fused proteins:**

1x PBS containing:

- 10 mM MgCl$_2$
- 0.5% Triton X-100
- 0.1% Non-Iodet P-40
- 5 mM DTT
- 10 mM PMSF
- 1 mg/ml Lysozyme
- 100 µg/ml DNase I

8) **Glutathione Beads elution buffer (pH-8.0):**

- 50 mM Tris pH 8.0
- 20 mM Reduced Glutathione
- 5 mM DTT
9) **TE (Tris-EDTA):**

- 10 mM Tris (pH-8)
- 1 mM EDTA (pH 7.5)

10) **Cytoskeleton Extraction Buffer (CSE)**

- 10 mM Tris-HCl, 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 Na₃VO₄

11) **Low pH antibody elution buffer:**

- 100 mM Glycine
- 150 mM NaCl
- 10 % Ethylene Glycol

pH was adjusted to 2.3 using HCl.
12) **Poly-L-Lysine - 0.1 %** (Sigma, Cat. no. P4707-50ml)

13) **Poly-D-Lysine hydrobomide** (Sigma, Cat. No. P6407) reconstituted in sterile glass distilled water to final conc of 0.05%, filtered using 0.22 μm syringe filter, and stored at 4°C.

14) **Resuspension medium for hippocampal neurons:**

   HAM’sF12 (Sigma, Cat. No. N6760) containing:

   - 1% Glutamax (GIBCO)
   - 10% Fetal Bovine Serum (GIBCO)
   - 5% Horse Serum (GIBCO)
   - 0.25% Ciprofloxacin (Cipla)

15) **Plating medium after transfection of hippocampal neurons:**

   The medium used for plating the neurons after transfection, consists of a mixture of HAM’sF12 and DMEM (1:1) supplemented with the following components:

   - 1% Glutamax (GIBCO)
   - 10% Fetal Bovine Serum (GIBCO)
   - 10% Horse Serum (GIBCO)
   - 0.25% Ciprofloxacin (Cipla)

   **Note:** DMEM from Gibco/Invitrogen, (Cat No. 11965-092) was found to be most suitable for neuronal growth. DMEM with HEPES adversely affects the viability of cells after transfection.
16) **Neurobasal medium:**

- Neurobasal medium (GIBCO)
- 1% Glutamax (GIBCO)
- 0.25% Ciprofloxacin (Cipla)
- 2% B27 Supplement (GIBCO 50x)

17) **Cell Lysis buffer for kinase assay:**

- 50 mM HEPES, pH-7.4
- 0.5 M NaCl
- 5 mM MgCl₂
- 0.5 mM EGTA
- 50 mM NaF
- 1% NP-40
- 1 mM Sodium ortho vanadate
- 1 mM PMSF

18) **Reaction buffer for kinase assay:**

- 50 mM Tris HCl, pH 7.4
- 10 mM MgCl₂
- 1 mM DTT
2.2 Plasmid Constructs used in the study:

1) GFP-PKCζ wild type and mutant:

PKCζ constructs were received as pCAN-HA human PKCζ-Wild type and pCMV-FLAG rat PKCζ-Kinase dead mutant from Steven Martin (University of California, Berkeley, USA). Wild type PKCζ was sub cloned into pEGFP-C2 vector using 5’ EcoRI and 3’ApaI sites. Similarly Kinase Dead mutant was cloned using EcoRI sites in pEGFP-C2.

Clone confirmation:

For GFP PKC Wt: The correct clone gives an insert release of 1.1 kb with BglII restriction digestion and 1.6 kb release with NdeI digestion.

For GFP PKC mutant: The correct clone gives an insert release of 1.3 kb with PstI digestion.

2) pcDNA-HA-PAR3:

SR-His-B-ratPAR3 (180 KD isoform) was received from Shigeo Ohno, Yokohama City University School of Medicine, Japan (JCB Izumi et al, 1998). The Par3 ORF was released from SR-His-B-ratPAR3 using NheI (end filled) and SalI sites and cloned into 5’EcoRV and 3’ XhoI sites of pcDNA-HA-C1 vector (generated in the lab previously).

Clone confirmation: Restriction digestion with HindIII releases the insert of 4.5 kb.

3) pcDNA-HA-PKCζ-N terminus:

pcDNA-HA-PKCζ-N terminus was made from pcDNA-HA-PKCζ-Wild type by ligating Xhol and XcmI end filled sites. It spans amino acids 10 to 265.

Clone confirmation: The correct clone gives an insert release of 0.7 kb with double digestion using EcoRI and XbaI.
4) **pcDNA-HA-PKCζ-C terminus:**

cpDNA-HA-PKCζ-C terminus was made by ClaI digestion and self-ligation of pcDNA-HA-PKCζ. It spans amino acids 233 to 592.

**Clone confirmation:** The correct clone gives an insert release of 0.7 kb with BamHI restriction digestion.

5) **pcDNA HA Dvl1:**

Human Dishevelled isoform 1 was received from Lin Li lab (Shanghai Institute for Biological Sciences, China). Full length Dvl1 was PCR amplified using the following primers and cloned into pGEMT easy cloning vector (Promega).

Fwd: 5' CCC GAA TTC ATG GCG GAG ACC AAA ATC ATC 3'

Rev: 5’ CAT GAT GTC CAC AAA GAA CTC 3

After sequence confirmation, it was released by EcoRI enzyme and cloned into EcoRI sites of pcDNA-HA-C1 (vector generated in the lab).

**Clone confirmation:** BglII and XhoI double digestion releases an insert of 1.9 kb in the correct clone.

6) **pBeta Actin-eGFP:**

pBeta Actin-eGFP was received as a kind gift from Prof. Garry Banker (Oregon Health and Science Centre, Oregon, USA)
7) **pEGFP-Dvl1:**

Human Dishevelled isoform1 received from LinLi lab was PCR amplified using the following primers:

Fwd: 5’ CCC GAA TTC ATG GCG GAG ACC AAA ATC ATC 3’

Rev: 5’ CAT GAT GTC CAC AAA GAA CTC 3’

The PCR product was digested with EcoRI and cloned into pEGFP-C2 vector using 5’ EcoRI and 3’ SmaI sites.

**Clone confirmation:** Restriction digestion by BamHI releases an insert of 0.8 kb.

8) **pEGFP-Dvl1-DIX:**

pEGFP-Dvl1-DIX was made by PCR amplification from pEGFP-Dvl1 using following primers:

Fwd: 5’ CCC GAA TTC ATG GCG GAG ACC AAA ATC ATC 3’

Rev: 5’ GGT GCT CTG CTC TGT GGA GCT 3’

This construct spans amino acids 2 to 210 of human Dvl1 and was cloned into pEGFP-C2 vector using 5’ EcoRI and 3’ SmaI restriction sites.

**Clone confirmation:** BamHI and EcoRI double digestion releases an insert of 0.6 kb in the correct clone.
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9) pEGFP-Dvl1-PDZ:

pEGFP-Dvl1-PDZ was cloned by PCR amplification from GFP-Dvl1 using following primers:

Fwd: 5’ CCC GAA TTC TCC TCT CGG CTA GTT CGG AAG 3’
Rev: 5’ TGG CAA CTG CAT GAC GCG GAC 3’

This construct spans amino acids 211 to 425 of human Dvl1, and it was cloned into pEGFP-C1 using 5’ EcoRI and 3’ SmaI sites.

Clone confirmation: Bam HI digestion releases 0.5 kb insert in the correct clone.

10) pEGFP-Dvl1-DEP:

pEGFP-Dvl1-DEP was cloned by PCR amplification from GFP-Dvl1 using following primers:

Fwd: 5’ CCC GAA TTC GAC TCA GGA CTG GAG ATC CGG 3’
Rev: 5’ CAT GAT GTC CAC AAA GAA CTC 3’

This construct spans amino acids 4426 to 695 of human Dvl1 and was cloned into pEGFP-C1 using 5’ EcoRI and 3’ SmaI sites.

Clone confirmation: BamHI and EcoRI double digestion releases an insert of 0.8 kb in the correct clone.

11) pCMV-myc-PAR6 isoform C:

pCMV-myc-hPar6 isoform C was received from Steven Martin (University of California, Berkeley, USA)
12) pGEX PAR3  712-936 (PKCζ substrate peptide):

The construct spans the region, which has been shown to be phosphorylated by PKCζ
in vitro (PAR3 amino acids 747–956, (Lin et al., 2000)). PCR amplification of this
fragment was done from SR-HisB-r-PAR3 (Shigeo Ohno) using the following
primers:

Fwd: 5’CCC GGA TCC CGC AGG ATC TCA CAC TCC3’

Rev: 5’CCC CTC GAG GGG CTT ATC GTA GGA CTT 3’

The PCR amplified product was digested with BamHI and cloned into BamHI and
SmaI sites in pGEX6P2.

Clone confirmation: Positive clone releases an insert of size 0.67 kb with BamHI and
XhoI double digestion.

13) His Dvl1 amino acids 593-695:

This fragment was generated by PCR amplification of the C-terminal region of Dvl1
for raising rabbit polyclonal antibodies against Dvl1. The primers used were:

Fwd: 5’ACT GGA GCT GGG GGT AGT GGC 3’

Rev: 5’CAT GAT GTC CAC AAA GAA CTC 3’

The PCR amplified fragment was cloned into EcoRV site in pET 30a after treatment
with T4 Polynucleotide kinase.

Clone confirmation: In the correct clone, restriction digestion with NcoI releases an
insert of size 300 nt, while the incorrect clone gets linearized.

Double digestion with DraIII and StuI releases an insert of 0.6 kb in the correct clone
while the incorrect clone gets linearized.
2.3 Antibodies used in the study:

The antibodies used in western blotting, immunoprecipitation and immunofluorescence analysis were mouse anti-PKCζ (H-2), rabbit anti-PKCζ (c-20), mouse anti-Dvl1, mouse anti-Dvl2 (c-2), rat anti-HA (Roche), mouse anti-GFP (B-2) (Santa Cruz Biotechnology), rabbit anti-PAR3 (Upstate), mouse anti-FXFG-containing nucleoporins (MAb414) and mouse anti-HA (Covance), mouse anti-Tau-1 (Chemicon). Rabbit polyclonal anti-Nup358 antibodies generated against a region spanning 2560-2971 amino acids of human Nup358 (Joseph et al., 2004), rabbit anti-GFP generated against full length GFP protein (Sahoo PK, 2012) and rabbit anti-Dvl2 generated against a region spanning aa 593-736 of mouse Dvl2, were available in the lab (Murawala et al., 2009).

2.4 METHODS:

1. Coating of coverslips for culture of hippocampal neurons:

One day before the isolation of neurons, glass coverslips were flamed quickly in the laminar hood, placed in 60 mm tissue culture grade petridish, covered with 3 ml Poly-L-lysine or Poly-D-lysine hydrobromide solution and incubated for 12 h in CO₂ incubator. The solution was removed and coverslips were placed under UV in laminar hood for 10 min. The coverslips were washed with plating medium once before plating the cells.

Note: The L-form of any amino acid is readily hydrolysed by cells; hence it is not suitable for coating the coverslips for long term cell cultures (7-15 days). In such cases Poly D-lysine is used as a coating alternative. For short term cultures (3-4 days), Poly-L-lysine, Poly-D-lysine and Poly-L-ornithine, all serve as equally efficient coating substrates. Use freshly coated coverslips for every experiment.
2. Isolation of hippocampal neurons:

Each pregnant Wistar rat was sacrificed on the 18th day of gestation by CO₂ asphyxiation. The abdomen was opened by making an incision on the ventral side. The two horns of the uterus were taken out. The pups were removed one by one from the uterus. The placental sac was opened and the umbilical cord was cut. The pup was placed on the working table, and without injuring the head, it was immobilized by inserting one pin in the snout, and another in the lumbar region. Fine forceps were used to peel away the scalp and the soft skull. The brain was then visible, and was separated from the brain stem, by scooping it out using a surgical spatula. The brain was kept on ice in a solution of cold 1x CMF PBS.

The brain was placed on the stage of a light-dissecting microscope in a glass petridish containing cold CMF-PBS. The occipital lobe was separated using curved forceps and was discarded, while the two cerebellar hemispheres were separated along the corpus callosum by a medial longitudinal section. Viewing the hemisphere from the inner face, the thalamus and the neighbouring structures were excised. The hippocampus then becomes visible as a thin, semi-circular structure. The meninges and the surrounding cortical tissue were removed using curved fine forceps. Thus, only the hippocampus was isolated (and kept in CMF-PBS on ice), and was further processed.

Note: Remove maximum extra tissue around the hippocampus to minimize the contamination of the culture by glial cells. The cells should not be left on ice for longer than 2-3 h, so isolation and processing should be done quickly and efficiently.

3. Trypsinization of hippocampal neurons:

The hippocampal tissue, stored in CMF-PBS on ice was pelleted down by centrifugation at 6000 rpm for 2 min. The supernatant was removed, 1 ml TPVG and DNAse enzyme (10 µg) was added and after gentle trituration, the cells were incubated at 37°C for 30 min. Later, 500 µl resuspension medium was added to cells, and resuspension was done gently.
After centrifugation at 6000 rpm for 3 min at room temperature, the supernatant was discarded to remove all traces of TPVG, and the process of resuspension and centrifugation was repeated three times. Cells were finally resuspended in 1 ml resuspension medium and cell counting was done using a Neubauer's chamber.

**Note:** Do not leave cells in TPVG longer than 30 min, they will die. Cut the end of 1 ml pipette tip before trituration, and repeat the dissociation step 3 times before plating, else they will grow in clumps. Do not over triturate, they will die.

### 4. Transfection of hippocampal neurons:

Transfection of hippocampal neurons was done using Amaxa NucleofectorII device. Briefly, 1 million cells were counted and collected in a sterile eppendorf tube. The supernatant was discarded and the cells were resuspended by gentle tapping. Transfection mix was made by adding 4 μg DNA and pBetaActin-eGFP (4:1), and 100 μl transfection reagent to the cells, which was mixed gently and transferred to the nucleofector cuvette. A pulse was given at program number G-013, and 1 ml resuspension medium was added immediately in the cuvette. Cells were then transferred to a sterile eppendorf tube and placed in the incubator for 10 min for recovery. Resuspension was done gently with 1 ml pipette tips with cut ends. Counting was done and finally 50,000 cells per well of 24 well plate were seeded onto Poly-L-lysine coated coverslips in 400 µl plating medium. The medium was replaced by neurobasal medium (reagent 16) after 6 h of plating the cells. After 36 h of plating, half of the medium was replaced by fresh neurobasal medium and the cells were analysed by immunostaining after 72 h of plating (3 days *in vitro*).

**Note:** The recovery step after transfection is very essential and must be done in a medium with low calcium. For this purpose, HAM'sF12 or RPMI can be used. Resuspension in medium containing high calcium (like DMEM) immediately after transfection results in drastic decrease in the cell viability.
5. Immunostaining of hippocampal neurons and non-neuronal cells:

The hippocampal neurons grown for 3 days in vitro were fixed using 4% PFA and 4% Sucrose in 1x PBS for 20 min at room temperature. Permeabilization was done using 0.2% Triton X-100 in 1x PBS for 10 min at room temperature. Primary antibody incubation was done in blocking buffer containing 1% NHS and 1% BSA in 1x PBS.

After 40 min of incubation, three washes were given using 1x PBS and indicated secondary antibody conjugated to Alexa Fluor dye (Invitrogen) was added in the blocking buffer along with Hoechst-33342 dye (Sigma-Aldrich) for visualization of DNA. Incubation was done for 30 min followed by three washes with 1x PBS and mounting in Vectashield Mounting medium (Vector laboratories). Coverslips were sealed using nail paint to avoid dehydration and observed under microscope.

COS7 cells were fixed with chilled methanol for 5 min and washed once with 1x TBS. A quick wash was given with 0.1% Triton X-100 in 1x TBS and primary antibody incubation was done for 30 min at room temperature in 2% NHS-TBS. Three washes were given with 1x TBS and secondary antibody incubation was done for 30 min in 2% NHS-TBS along with Hoechst-33342 dye. Mounting was done in Vectashield mounting medium.

Images of hippocampal neurons were obtained with a Zeiss Axiovert 200M using a Plan Apochromat 63x NA1.4 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 of COS7 cells were obtained using a laser-scanning confocal microscope (TCS SP5; Leica). Images were processed further in Adobe Photoshop CS3.
6. The criteria used for counting of hippocampal neurons:

The neurons were scored on the basis of the following criteria:

i) Neurons with short neurites around the cell body, which were of similar length and Tau-1 negative, were termed as **no axons**.

ii) Neurons with a single long neurite, which is Tau-1 positive and at least two times longer than the other neurites around the cell body, were termed as **single axon**.

iii) Cells which had two or more neuritis, which were Tau-1 positive and two times longer than other neurites, were termed as **multiple axons**.

7. Transfection in HEK293T cells:

For transfection using Poly Ethylene Imine (PEI), 24 h prior to transfection, the cells were seeded in a 6 well plate. During transfection, 2 μg DNA was mixed with 100 μl Opti-MEM or serum free DMEM, while 10 μl PEI (4-5 μl/ μg DNA) was mixed with 100 μl Opti-MEM separately. After 5 min incubation, both were mixed and incubated again for 20 min at room temperature (RT). The old plating medium was removed from the cells and replaced with 1 ml fresh DMEM containing 1% FBS. After 12 h of transfection, 1 ml DMEM containing 10% FBS was added in the plate. After 12 h of adding fresh medium (that is 24 h after transfection), complete medium was replaced with 2 ml fresh DMEM containing 10% FBS and cells were placed in the incubator for 12 h. The cells were processed 36 h after transfection.

**Note**: low serum helps in obtaining better transfection efficiency using lipid based transfection reagents.
8. Immunoprecipitation:

For immunoprecipitation analysis, the cells were washed with ice-cold 1x TBS once and lysed in chilled cytoskeleton extraction (CSE) buffer with inhibitors (mammalian protease inhibitor cocktail without EDTA [Roche Applied Science]). To make the cell lysate, cells were resuspended in CSE buffer and subjected to mild sonication for 2 pulses at 70% amplitude. The lysate was centrifuged at 12000 rpm, 30 min at 4 ºC to remove cellular debris. The total protein in the lysate was calculated using Bradford assay reagent (Biorad) and up to 500 μg total protein was taken for immunoprecipitation analysis. For preparing total protein sample for western blot, the supernatant was mixed with equal volume of 3x PAGE loading dye, and boiled at 95 ºC for 3 min. For immunoprecipitation, 20 μl sepharose beads (n-Sepharose4 Protein A beads, GE-Healthcare) were bound with 5-10 μg respective antibodies. Incubation of antibodies and beads were done in 1x TBS at room temperature, on rotospin for 2 h. Conjugated beads were washed with 1 ml lysis buffer, and incubated with the cell lysate at 4 ºC for 2 h. In case of HA pull downs, commercially available EZview Red anti-HA beads (Sigma) were directly used for immunoprecipitation after 1 wash with 1x TBS. EZview Red Protein-G beads were used as control in these experiments. The immunoprecipitates were washed two times with the CSE lysis buffer and once with 1xTBS, eluted in SDS dye and separated on SDS-polyacrylamide gels. The proteins were transferred to PVDF membrane (Millipore) using semi-dry transfer apparatus (Biorad or Amersham), and western blotting was performed using indicated antibodies.

For western blotting, PVDF membranes were incubated with specific primary antibodies in either 1% BSA in TBST (for mouse monoclonal antibodies) or 0.5% non-fat dry milk (Amul) for 90–120 min, washed with 1x TBST for three times and incubated with HRP conjugated secondary antibodies at 1:10,000 dilution. Further membranes were washed three times with 1x TBST and developed using ECL plus western detection kit (GE Healthcare), following the manufacturer’s instructions.
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9. RNA Interference:

For RNA interference experiments in HEK293T, the cells were seeded in a 24 well plate, 12 h prior to transfection. The transfection was done with oligofectamine (Invitrogen) according to the manufacturer’s instructions. Briefly, 60 nM annealed siRNA was mixed in 50 µl Opti-MEM while 3 µl oligofectamine was mixed with 50 µl Opti-MEM separately. After 5 min of incubation, the contents of the two vials were mixed and incubated for 20 min at room temperature. The complex was added to cells with fresh 500 µl DMEM containing 1% FBS. After 12 h, the medium was changed to DMEM containing 10% FBS. The cells were lysed and analysed after 64-72 h of transfection. The oligonucleotide sequences used for silencing of the target gene were as follows (Murawala et al., 2009):

1) Control (non-silencing) siRNA, 5’-AATTCTCCGAACGTGTCACGT-3’ (Qiagen or Santa Cruz Biotechnology)

2) Nup358 siRNA, 5’-AAGGTGAAGATGGATGGAATA-3’ (Dharmacon/Qiagen)

10. *in vitro* Kinase assay for PKCζ activity:

This assay was performed to access the activity of PKCζ using GST-tagged fragment of PAR3 (aa 712-936, construct 13) as a substrate. GFP tagged PKCζ was overexpressed in HEK293T cells and the cell lysis was done after 36 h of transfection using a specific buffer as mentioned in materials section (reagent18). Immunoprecipitation was done using protein A/G beads (Santa Cruz Biotechnology) bound with rabbit polyclonal antibodies against GFP. The immunoprecipitates were washed once with 750 µl lysis buffer, followed by one wash with 750 µl TBS, and finally with 500 µl kinase assay buffer (reagent 19). Ten percent of the total beads taken for IP were separated for western blotting and boiled with 3x SDS PAGE loading dye at 95 °C for 3 min. Rest of the beads were incubated with 5 µg of purified GST tagged substrate (PAR3 amino acids 747-956) and 10 µCi γ-32P-ATP for 5 min at 30°C. A myristoylated PKCζ pseudosubstrate (Sigma-Aldrich) was used as a control in this experiment.
The beads (after pull down) were incubated with 20 µM pseudosubstrate for 20 min on ice before adding the PAR3 substrate in the reaction mix. Phosphorylated substrates were resolved on SDS–PAGE and visualized by autoradiogram.

11. Purification of GST tagged PAR3 for in vitro PKCζ kinase assay:

GST tagged PAR3 (712-936 amino acids) was transformed in E. coli BL21 Codon plus, inoculated in 500 ml LB broth (HIMEDIA) and grown at 37 ºC for 2-3 h to a density A<sub>600</sub> = 0.5-0.6. For induction of the protein, IPTG (MP Biomedicals or Sigma) was added to a final concentration of 0.5 mM and incubation was done at 37 ºC for 5 h. After induction, the culture was pelleted down in Sorvall centrifuge at 8000 rpm, 4 ºC for 10 min. The cell pellet was resuspended in 50 ml cold lysis buffer (Reagent no. 6), and lysed by sonication, keeping on ice throughout. The cell debris were pelleted by centrifugation at 12000 rpm, 4 ºC for 30 min. The supernatant was incubated with 1 ml (bed volume) glutathione beads, pre washed with lysis buffer, for 2 h at 4 ºC. After the binding, the solution was added to econocolumn (20 ml BioRad), and the lysate was allowed to flow through it, leaving only the beads in the column. The beads were washed by adding 10 ml lysis buffer twice and 5 ml 1x TBS once. The elution was done by adding 1 ml cold Glutathione beads elution buffer (Reagent 7), incubating on ice for 5 min and collecting the flow through in eppendorf tubes. The fractions obtained were analysed for protein amount by SDS PAGE and the fractions containing significant amount of protein were pooled, dialysed in 1x TBS and then concentrated in Amicon ultra columns (10 kDa cut off, Millipore). Purified proteins were stored in aliquots of 50 µg in -80 ºC freezer.

Note: Always check the induction before proceeding with large-scale purification of proteins. For that purpose, remove 1 ml culture prior to addition of IPTG and keep in an eppendorf tube for the same time and temperature as the induced culture. Collect 1 ml culture from the large scale induced sample and process along with the un-induced sample, check induction by SDS PAGE followed by coomassie staining.
12. Purification of His tagged Dvl for antibody purification:

His tagged Dvl1 fragment (aa 593-695) was transformed in *E. coli* BL21, inoculated in 500 ml LB broth and induced with 0.5 mM IPTG at 20 °C for 6 h. The processing was done according to the method mentioned above (method no. 11), with the exception of the lysis buffer (reagent no. 4), Nickel beads for purification, and elution buffer (reagent no. 5).

**Note:** The bacterial proteins sometimes bind non-specifically with the Ni beads, and get eluted along with the protein of interest. To remove the non-specific proteins, one wash with 1x TBST was given after the incubation of the lysate and beads followed by washes with the lysis buffer and 1x TBS.

13. Generation of antibody against Dvl1:

For the purpose of antibody generation, 250 µg His tagged Dvl (amino acids 593-695) was emulsified with Freund’s complete adjuvant (Sigma-Aldrich) and injected in Newzealand White rabbit (8 months old) intradermally. Before injection of the antigen, 10 ml blood was drawn to collect the pre-immune serum. After one month of the injection of the antigen, 125 µg antigen emulsified with Freund’s incomplete antigen was injected subcutaneously in the rabbit. Blood was collected after 10 days and serum was isolated for purification of antibodies. The rabbit was given booster doses of the antigen coupled with Freund’s incomplete antigen and the serum was stored in -80 °C freezer until further processing.
14. Affinity purification of Dvl antibody from rabbit serum:

For the purpose of affinity purification of the antibody, Dvl antigen was purified as described above, dialysed against HEPES (80 mM, pH 7.5) and concentrated to 1 mg/ml. As a pre-bound sample, 5 µl of the purified protein was mixed with 3x SDS dye and boiled at 95° for 3 min. Affigel 10 (BD Biosciences) was taken in a column (0.5 ml bed volume), washed quickly with chilled glass distilled water and incubated with 3-4 mg antigen for 2 h at 4 ºC. The flow through after antigen incubation was collected and 5 µl was taken for checking the efficiency of antigen conjugation.

The pre and post bound antigen samples were checked by SDS PAGE and coomassie staining. The column was washed 3 times, alternatively with 1x TBS and low pH antibody elution buffer (reagent 10). To remove any unbound antigen, and to ensure that the pH of the column is neutral; two more washes were given with 1x TBS.

The serum (5 ml) was diluted 1:1 with 1x TBS and incubated with the beads prepared as above and kept on rotospin at 4 ºC for 4 h. Meanwhile 100 µl of 2 M Tris (pH 9) was taken in 10 eppendorf tubes and kept ready for collecting the antibody fractions. After incubation, the column was washed with 1x TBST once and 1x TBS twice. The antibody fractions were collected by adding 1 ml of low pH antibody elution buffer (reagent 10) to the column and immediately collecting the flow through in the eppendorf tubes containing 2 M Tris (pH 2.3). The column was washed with 1x TBS and stored in 20% ethanol at 4 ºC for future use. The fractions were checked for the amount of antibody purified by running on SDS PAGE with a known amount of BSA standard protein followed by coomassie staining. All the fractions having significant amount of antibody were pooled, dialysed against 1x TBS and concentrated upto 0.5-1 µg/µl in Amicon Ultra column (Millipore, cut off 10 kDa). The antibody was stored in -80 ºC freezer in aliquots of 50 µl. The working aliquot was mixed with glycerol (Sigma-Aldrich) and stored in -20 ºC freezer.
15. Preparation of Wnt5a conditioned medium:

Wnt5a condition medium (W5a CM) was prepared from a Mouse-L cell line stably expressing the Wnt5a gene (kindly provided by Prof. Roel Nusse, Stanford University, CA, USA). For preparing the control conditioned medium (CCM), Mouse-L cells (ATCC, USA) were used. The conditioned medium was collected by growing control and Wnt5a-Mouse-L cells in 150 cm$^2$ T flasks (BD biosciences) up to 80% confluency in DMEM containing 10% FBS. The medium was then changed to DMEM containing 0.1% FBS. and after 3 days, the medium was collected and fresh DMEM containing 0.1% FBS was added in the flask. After another 3 days, the second batch of medium was collected. Both the batches were mixed and filtered through 0.22 μm filter. The medium was aliquoted and stored in -80 °C freezer. For use in experiments, the control CM and W5a CM were mixed with fresh DMEM with 0.2% FBS in 1:1 ratio and was added to cells for the indicated time. Presence of active Wnt5a in the conditioned medium was checked by western blotting for the shift in the mobility of Dvl2 by SDS PAGE and western blotting.