Chapter 2

Materials and methods
Bacterial Strains

DH5α, JM109, BL 21 DE3

Cell lines

PCC4, HeLa, C3HT105, SW260, Colo 205.

Cell Biology Protocols

Cell culture

Embryonal carcinoma cell line PCC4 and cervical cancer cell line Hela (ATCC; Rockville, MD) were maintained as monolayer culture in DMEM with 10% fetal calf serum (Sigma) at 37°C in 5% CO₂ atmosphere (Bisht et al., 1994). Exponentially growing cells were seeded and cultured for 16 h prior to induction of differentiation. Differentiation of PCC4 cells was induced by heat shock as described earlier (Bisht et al., 1994).

Immunofluorescence

HeLa/ PCC4 cells were cultured on glass coverslips for 24 hr and either untreated or differentiated were processed as described earlier (Batth et al., 2001). Cells were fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 for 10 min. Coverslips were blocked in 5% non-immune serum from the same species as the labeled secondary antibody. Cells were incubated with affinity-purified anti-Cul4B antibody (1:100 dilution) in phosphate buffer saline containing 5% non-immune serum from the same species as the labeled secondary antibody at room temperature for 1 h. The cells were washed and incubated with FITC conjugated secondary antibody (1:2000) for 45 minutes, then washed in phosphate buffer saline and then mounted in mounting medium containing DAPI (counter stain) and examined by confocal microscopy.
Transfection (Invitrogen lipofectamine experimental manual)

Transfection into mammalian cells was done using lipofectamine as described by the suppliers. For PCC4 as well as C3H cells transfection was done when cells were about 50% confluent. 1μg -1.2μg DNA was transfected per well in a six well plate. Plasmid DNA was pre-complexed with the 4μl of lipofectamine plus reagent in the presence of 50μl of medium and incubated for 15 minutes. In another tube 3μl of lipofectamine was diluted with 50μl of medium and mixed with DNA-LF+ mix and incubated for 15 minutes at room temperature. The growth medium of cells was replaced with 700μl of serum-free medium before starting the transfection procedure; DNA lipid complex was added to the cells and kept at 37°C in CO₂ incubator for 3 hours. After 3 hours, the mix was replaced with growth medium. In the case of PCC4 cells, transfection efficiency was 90% and in the case of C3H cells it was 70%.

Clonal selection of stable transfectants (Invitrogen technical manual)

Prior to selection of stable clone G418, the dose response was checked for the cells. In the case of PCC4 cells, it was found to be 600μg/ml. This was the G418 concentration at which non-transfected cells died within 5 days, wherein the G418 medium was changed every 3rd day. To get stable clones, transfection was done as described in the methods section. Cells were grown for 24 hours following transfection and then the growth medium was replaced with fresh growth medium containing the required amount of G418. G418 medium was replaced every 3-4 days. Once the clones of resistant cells started growing, separate clones were selected, expanded and checked for expression by western blot.
**Drosophila Protocols**

Fly genomic DNA isolation (As described at http://www.fruitfly.org)

30 anesthetized flies were collected in an eppendorf tube and frozen at -80°C. The flies were homogenized in 200-µl of bufferA (100mM Tris-HCl, pH 7.5, 100mM EDTA, 100mM NaCl, 0.5% SDS) until only the cuticle remained. The sample was incubated at 65°C for 30 minutes. 800µl of LiCl/Kac (One part 5M Kac solution: 2.5 parts 6M LiCl stock) solution was added and the sample was incubated on ice for 10 minutes. It was then centrifuged at room temperature for 15 minutes. The supernatant was transferred to a new tube and 600µl of isopropanol was added. The sample was centrifuged for 15 minutes at room temperature. The supernatant was discarded and the pellet was washed with 70% ethanol, dried completely and resuspended in 150µl of TE (pH 8.0).

Inverse PCR (As described at http://www.fruitfly.org)

To identify the correct insertion position of the transposon, genomic DNA was isolated from the mutant fly as described in the methods section. Two separate reactions were set up to digest the genomic DNA of two flies (10ul) separately with SAU3A1 and MSPI in the presence of 100µg/ml RNase and NEB buffer2 in 25µl reaction volume each. Samples were incubated at 37°C for 2.5 hrs and then heat-denatured at 65°C for 20 minutes. 10 µl of digested DNA was ligated in a 400-µl-reaction mix in the presence of 2 Weiss units of ligase and 40 µl of 10X ligase buffer, at 4°C, overnight. Ligated DNA was ethanol precipitated, dried completely and resuspended in 150µl of TE. PCR reaction was set up with the appropriate primers for the P-element and primer for the gene of interest. 10 µl of ligated genomic DNA, 1 µl of 10µM reverse and 1µl of forward primers. 2µl of 2mM dNTP each, 5µl of 10X taq buffer with MgCl₂, 2 units of taq and double distilled water to make the volume up to 50 µl. PCR reaction was done using the following parameters:
95°C for 5 min, 95°C for 30 seconds, primer annealing temperature for 1 min and 68°C for two minutes for 35 cycles, 72°C for 10 min, 4°C hold. The PCR product was run on the 8% agarose gel, specific amplified product was eluted from the gel and given for sequencing.

**Immunostaining of imaginal discs** (Xu and Rubin, 1993, with modification)

Larvae (3rd instar) were dissected and inverted in PBS. Fixing was done for 20 min in 4% (w/v) paraformaldehyde in PBS. Tissues were blocked in 1% (w/v) BSA, 0.3% (w/v) TritonX-100 in PBS for 1 h at 25°C. The primary antibody was added at recommended dilution. After overnight incubation at 4°C, discs were washed three times in PBS containing 0.3% (w/v) Triton X-100 and incubated with Alexa secondary antibody (Molecular probes) 1:1000 dilution for 1 hour. Discs were dissected out from the larvae under light microscope and mounted into the glycerol containing DAPI.

**Cuticle Prep** (Krause lab protocol, http://www.ceolas.org/VL/fly/protocols.html)

Embryos from CS and mutant flies were collected on embryo collection medium containing plates and allowed to age for 24-36 hour. Larvae were collected and incubated at 60°C for 10-12 hours with 1:4(Glycerol: Acetic acid). Larvae were spread on a slide and mounted with Hoyer’s mounting medium and incubated at 60°C overnight for clearing of larvae.

**Pre-absorption of antibodies**

Embryos were collected on embryo collection medium containing plates for 14-16 hr, then pooled in a home made basket, washed with water and bleached with 50% bleach for 3 minutes. The embryos were rinsed with water and transferred to a scintillation vial containing 1ml of n-heptane; 1 ml of 4% paraformaldehyde was added to it. The vial was incubated for 20-30 minutes. The bottom layer containing paraformaldehyde was removed
and embryos were rinsed in heptane twice, devitalized by shaking them hard for 30 seconds to one minute with 1:1 ratio of n-Heptane and HPLC grade methanol. Embryos were rinsed with methanol 2-3 times and stored at -20°C till further use. Embryos were rehydrated by rinsing with PTX (PBS, 0.3% tritonX-100) and then incubating with PTX for 15 more minutes. Blocking was done with PBTX (PTX with 0.1% BSA) for 2-3 hours. The final blocking was done by incubating a 1:10 dilution of antibody with the embryos overnight at 4°C. Pre-absorbed antibodies were stored as 1/10 or 1/20 dilution at 4°C for several weeks with azide.

**Immunostaining of Embryos** (Peifer et al, 1990, with modifications)

Dehydrated embryos stored in methanol were washed with PTX and processed as described above until the to blocking procedure. Embryos were incubated with the required dilution of primary antibody overnight at 4°C, washed with PTX for 2 hours with 2-3 changes, incubated with 1:1000 dilution of secondary antibody (alexa) for two hours, washed for two hours with 2-3 changes and mounted in glycerol with DAPI. Whenever required, the embryos were flattened with the help of a needle and mounted.

**LacZ staining of embryos** (Su et al. 1998)

Embryos were collected as described above and washed twice with NaCl TritonX-100. The embryos were dechorionated with 30% bleach for 90 seconds, rinsed with NaCl TritonX, washed with double distilled water and incubated with 5ml 4% paraformaldehyde and 5ml n-heptane, for 20 minutes with shaking. The upper layer of PF was removed; water was added and mixed vigorously. Water and then heptane were removed completely. Embryos were rehydrated by shaking them with NaCl, tritonX -100 in a glass vial for 5 minutes. NaCl, TX was discarded, X-gal was added and embryos were incubated at 37°C for 30 min to 1 hr (till color developed). The x-gal was removed, pre-warmed n-heptane (at
50°C) followed by an equal volume of pre-warmed methanol (50°C) were added and vortexed vigorously for 20 seconds. Heptane was removed completely and rinsed with methanol 2-3 times. Embryos were stored at -20°C in methanol till used.

Molecular biology protocols

Preparation of competent cells

The competent cells were prepared according to Hanahan (1985) with minor modifications. A single colony of *E. coli* DH5α or BL21 was inoculated in 1ml of LB and incubated overnight. The culture was inoculated in 100 ml of LB (1:100 dilution) medium and incubated at 37°C with vigorous shaking till optical density at 550nm reached 0.35 (2-3x 10^7 cells / ml). The culture was chilled on ice for 15 min and the cells were pelleted by centrifugation at 2000 rpm for 12-15 min at 4°C. The supernatant was drained thoroughly and cells were resuspended in 0.33 volume of RF1. After chilling on ice for 15min, cells were pelleted as before, resuspended in 0.08 volume of RF2 and left on ice for another 15 minutes. Aliquots (200µl) of these cells were flash frozen in liquid nitrogen and stored at -70 °C till needed.

Transformation

The frozen competent cells were thawed on ice slowly just before use. Transforming DNA in a volume less than 10 µl was mixed with competent cells and incubated on ice for 15 minutes. The cells were subjected to heat shock for 90 seconds at 42 °C, followed by rapid chilling on ice for 5 minutes. An aliquote of 800µl of LB medium was added to the cells and the cells were incubated for an hour at 37°C with gentle shaking. When supercoiled DNA was used for transformation, 50-100µl mix was plated and for a ligated mix, the entire mix was pelleted at 4000 rpm for 2 minutes, the supernatant was discarded, pellet
was suspended in 100 μl of LB medium and plated on an LB plate containing required antibiotic and color development reagents (IPTG 10mM and X-Gal 20mg/ml). The plates were incubated overnight at 37°C and recombinants were picked up. The efficiency of transformation was routinely above $10^6$ colonies per μgm of super coiled DNA used.

**Large Scale Plasmid prepration**

Plasmid DNAs were prepared by alkaline lysis method (Brinboim and Doly 1979) described by Sambrook et al. (1989) with minor modifications. The bacterial pellet from an appropriate culture was washed in 10 ml of STE (0.1 M sodium chloride, 10mM tris-HCl pH 8.0, 0.1 mM EDTA) and the cells were resuspended in 10 ml of GTE (50mM glucose, 25mM tris-HCl pH 8.0, 10mM EDTA) containing 10mg of lysozyme. The contents of the tubes were mixed gently and incubated at room temperature for 10 minutes. 20 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added, mixed gently by swirling the tubes, and incubated on ice for 10 minutes. 15 ml of ice-cold solution III (3M potassium, 5M acetate solution cf pH 4.8) was added to the above and mixed by inverting the tubes. The contents were incubated on ice for 15 minutes. Genomic DNA and bacterial debris were pelleted at 8000 rpm at 4°C in Sorvall SS34 rotor for 30 minutes. The plasmid DNA in the supernatant was precipitated by adding 0.6 volume of isopropanol at room temperature for 30 minutes. Plasmid DNA was pelleted at 10,000 rpm for 30 minutes at room temperature. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 8 ml of T.E. (10mM Tris-HCl pH 8.0, 0.1 mM EDTA).
Small Scale isolation of plasmid DNA using diatomaceous earth method

This inexpensive and simplified protocol, based on Hansen et al., 1995, gives high yields of plasmid DNA and also assures high purity. It is suitable for cloning, PCR, sequencing, site-directed mutagenesis and *in vitro* transcription, etc.

An *E. coli* culture (3ml) was grown overnight (at least 16 hrs) in LB medium containing an appropriate antibiotic (e.g. ampicillin 25-50 µg/ml). Cells were harvested by centrifugation for 2 min and suspended in 300-500µl of Suspension Solution at room temperature. 300 - 500 µl of Lysis Solution was added, mixed very gently and kept at room temperature for about 5 min (but no more than 5 min). 300 - 500 µl of Neutralizing Solution was added to the tube, it was inverted gently several times and centrifuged for at least 7 min. Fresh diatomaceous columns were prepared during this time (preparation of columns is mentioned later). Although diatomaceous solutions store well, the columns don't. The supernatant was transferred carefully and mixed with approximately the same volume of binding buffer in a syringe and the mixture was applied to a freshly made diatomaceous earth column. Once all the solution was transferred, gentle suction was applied in the same manner as described for preparation of the column. 1 ml of Washing Solution was added and gently drained in the same way. The column was then placed in an eppendorf tube and centrifuged for at least 3 min to make sure that all the Washing Solution has been completely removed from the column. It is necessary to repeat this step twice to obtain high quality plasmids. The washed and drained column was placed in a new eppendorf tube and 50 µl of pre-heated MQ water (70 - 80 °C) or TE buffer (at room temperature) was added to elute the DNA and placed at room temperature for a maximum of 10 min. The column was centrifuged for 1 - 2 min. This step was repeated two or three times to elute virtually the entire DNA.
Solutions required:

1. **Suspension Solution**

50 mM Tris-HCl, pH 7.5 - 8.0, containing 10 mM EDTA and 100 µg/ml DNase-free RNase A.

Store at 4°C.

2. **Lysis Solution**

0.2 M NaOH containing 1 % SDS

3 M Neutralising Solution

4 M potassium-acetate, pH 4.8

4. **Binding Buffer**

6 M guanidine hydrochloride

It is not necessary for guanidine hydrochloride to be dissolved in TE buffer as described in Hansen et al. (1995) as MQ water is equally good. 5 M or 4 M works well but is preferable. Anything less than 3 M gives poor results.

5. **Washing Solution**

80% isopropanol (dilute to 80 % with MQ water). Ethanol is generally good as a washing solution, except that isopropanol is cheaper.

6. **TE Buffer**

10 mM Tris-HCl, pH 8.5, containing 1 mM EDTA

7. **Diatomaceous Earth Solution**

The preparation of this solution is crucial. The diatomaceous earth (Sigma D-5384 or other brands) was suspended at 50 mg/ml in water and left to sediment for more than 3 hrs. As much of the water containing the white gelatinous colloidal suspension as
possible was carefully discarded, leaving the sediment intact. This was repeated at least 3 times. If fine gelatinous matter was found during use, then the supernatant was carefully discarded and replaced with the same amount of water to maintain the same concentration of diatomaceous earth as above. Again, any milky suspension of diatomaceous earth was removed as described above.

The diatomaceous earth solution was resuspended thoroughly before use. A 2 - 5 ml syringe was used as a minicolumn and attached to a vacuum fitting. 500 - 600 µl (25 - 30 mg) of diatomaceous earth solution was loaded on to the column and suction was applied. Once all the solution was loaded, the column was monitored from above and gentle suction was applied. The vacuum was disconnected immediately after the liquid phase disappeared and the surface became solid. The column looked greyish white at this stage, with a thin brilliant white band at the bottom. The column could also be reused after appropriate cleaning as described below.

1) The diatomaceous earth was completely removed from the column.
2) The column was soaked in 0.1 M HCl for at least 1h and boiled for 10-20 min.
3) It was washed thoroughly using MQ water or distilled water and autoclaved.
4) A filter was fitted in the column using a yellow micropipet tip prior to use.

**RNA Isolation by Trizol Method (TRIZOL instruction manual)**

As PCC4 are attached cells, TRIZOL was added to the dish (1ml/10cm²) and cells were scraped and passed through the pipette several times. The tubes were incubated for 5 minutes at room temperature. 0.2 ml of CHCl₃/1ml of trizol was added and the tubes were shaken vigorously by hand for 15 seconds and incubated at 15-30°C for 2-3 minutes. The
samples were centrifuged at 12000Xg for 15 minutes at 2-8°C. The aqueous phase was collected and 0.5 ml of isopropanol was added per ml of trizol. Samples were incubated at 15-30°C for 10 minutes and centrifuged at 12000xg for 10 minutes at 2-8°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol by disrupting the pellet in 1ml ethanol per ml of trizol used initially and centrifuged at 7500Xg for 5 minutes at 2-8°C. The pellet was collected, dried completely and dissolved in DEPC treated water.

**Estimation of nucleic acid concentration**

The concentration of nucleic acid was estimated by measuring the O.D. at 260 nm. An empirical relationship used for this purpose was that O.D.1 is equivalent to 50 µg of double stranded DNA and 40µg of RNA/ml. Purity of DNA and RNA was checked by taking the ratio of A260/A280. Pure RNA, and DNA will have A260/A280 absorbance ratio of approximately 2 to 1.8.

**Differential Display Analysis**

DD play was done as described by Liang et al (1994a, 1994) using GenHunter DD Play kit. The whole procedure can be divided into 4 steps

**DNAse treatment of RNA**

Reverse transcription using anchored oligodT primers

PCR amplification

Denaturing Polyacrylamide Gel electrophoresis

DNAse treatment of RNA

Prior to use of total RNA for DD PCR, the RNA was treated with DNAse to remove possible contaminant genomic DNA. 10-50 µg of RNA was treated with 10 units of DNAse in the presence of reaction buffer (100mM Tris-HCl, pH 8.4, 500mM KCl, 15mM
MgCl₂ and 0.01% gelatin) at 37°C for 30 minutes. The sample was extracted with phenol:CHCl₃ to remove the DNAse. 40μl of phenol:CHCl₃ (3:1) was added and the sample was vortexed for 30 seconds. The sample was incubated for 10 minutes on ice, centrifuged at maximum speed in an eppendorf centrifuge at 4°C for 5 minutes. The aqueous phase was collected and 5μl of sodium acetate and 200 μl of 100% ethanol were added to it. The sample was kept at -80°C for 1 hour and centrifuged for 10 minutes at 4°C. The supernatant was removed carefully and the RNA pellet was washed with 0.5 ml of 70% ethanol in DEPC-H₂O. Ethanol was removed completely by centrifugation, first for 5 minutes, and then again briefly to remove the residual liquid. RNA was redissolved in 10-20 μl of DEPC-H₂O.

Reverse transcription using anchored oligoT primers

Different RNAimage kits are available from Genhunter. The kit used in this study contains three anchored primers (described in the section on primers) H-T₁¹G, H-T₁¹C, H-T₁¹A and 8 arbitrary primers. RNA was isolated from the cells, treated with the RNAse free DNAse and the integrity and purity of the RNA was checked by denaturing agarose gel electrophoresis and by measuring the optical density as described above. The reaction was set in 0.2 ml thin walled PCR tubes with 2μl of 0.1 μg of freshly diluted RNA sample, 1.6 μl of 250-μM dNTP, 2.0μl of 2μM anchored primer, 4μl of 5X RT buffer (125mM Tris-HCl, pH 8.3, 188mM KCl, 7.5 mM MgCl₂, 25 mM DTT) and 9.4μl of water. Samples were kept in a thermal cycler programmed to 65°C for 5 minutes, 37°C for 1 hour, 75°C for 5 minutes, 4°C for 2hours. After the tubes were maintained at 37°C for 10 minutes, the program was paused, 1μl of MMLV reverse transcriptase was added to the tubes and the program was restarted. On completion of the program, tubes were spun briefly and kept on ice for PCR or stored at -20°C for later use.
PCR amplification

PCR was carried out using one of the arbitrary primers and the same anchored primer as used for RT-PCR. The PCR reaction was set in thin walled PCR tubes by adding 10µl of water, 2µl of 10X PCR buffer (100mM Tris-HCl, pH 8.4, 500mM KCl, 15mM MgCl₂ and 0.01% gelatin), 1.6-µl of 25µM dNTP, 2µl of 2µM arbitrary primer, 2µl of anchored primer, 0.2 µl of α-³³P dATP and 1 unit of taq DNA polymerase. For amplification, the thermal cycler was programmed at 94°C for 30 seconds, 40°C for 2 minutes, 72°C for 30 seconds, for 40 cycles, followed by 72°C for 5 minutes, 4°C.

Denaturing Polyacrylamide Gel electrophoresis

6% polyacrylamide gel with 8M urea was prepared in TBE buffer (for 1 litre TBE buffer, 108g of Trizma base, 55g of boric acid, 3.7g of EDTA free acid). The gel was left for polymerization for 2 hours before the run. It was then pre-run for 30 minutes. Urea was completely flushed from the wells just before loading. 3.5 µl of each sample was mixed with 2µl of loading dye (95% formamide, 10mM EDTA, pH8.0, 0.09% xylene cyanol and 0.09% bromophenol blue) and incubated at 80°C for two minutes, immediately before loading to the gel. Samples were electrophoresed for 3.5 hours at 60 watts constant power (with voltage not exceeding 1700) until the slower moving dye reached the bottom. Gel was blotted on a piece of 3M paper, covered with plastic wrap and dried using a gel dryer at 80°C for 1 hour under vacuum. An autoradiogram was oriented with the dried gel using stapler punches. After developing the film (overnight-72 hour exposure), it was oriented with the gel, the band of interest was punched through the film with a needle at four corners, the film was removed and the band of interest was cut out using a clean razor.
gel fragment was soaked in 100μl of dH2O along with the 3M paper for 10 min. The sample was boiled for 15 min with the cap tightly closed with parafilm, tubes were spun for two minutes and the supernatant was transferred to new tube. 10μl of 3M sodium acetate, 5μl of glycogen (10 mg/ml) and 450μl of 100% ethanol were added and the tubes were kept in -80°C freezer for 30 min. Subsequently, the tubes were spun for 10 minutes at 4°C to pellet the DNA. The supernatant was removed and the DNA pellet was washed with 200μl of 85% ethanol; ethanol was removed completely by spinning briefly and the pellet was dissolved in 10 μl of water. 4μl of it was used for re-amplification.

**Random primer labeling of DNA**

Double stranded DNA (50-200ng) was denatured in a boiling water bath for 2 minutes, followed by rapid chilling on the ice. To this DNA, 5μl of 10X labeling (500mM tris-HCl pH 7.2, 100mM MgSO4, 1mM DTT) buffer, 2μl of nucleotide mix containing 300μM of each nucleotide except the one selected for labeling, 2μl of 10mg/ml nuclease free BSA, 50μCi of α-32P dNTP and 5units of DNA polymerase large (klenow) fragment, were added. The final volume was made up to 50μl and incubated at 37°C for 2-3 hours. The reactions were stopped and free radiolabelled nucleotides were removed by spun column chromatography as described below.

**Sephadex G-25 column chromatography**

Sephadex G-25 spun column chromatography was used to separate free radiolabel from that which was incorporated in the probe. A sterile 2 ml plastic syringe was plugged with sterile glass wool. The syringe was filled with autoclaved G-25 slurry in TE and the column was centrifuged at 4000 rpm in a Remi tabletop centrifuge for 3 minutes at room temperature. The column was then equilibrated for the desired volume (200μl) by repeated
loading of the fixed volume of TE and centrifuged till the volume of flow through was equal to the volume loaded. After equilibration, the DNA to be purified was loaded on to the column and centrifuged for 3 minutes at 3000 rpm. The flow through contained the labeled probe; free radiolabel as well as very small-labeled fragments remained in the column.

**Norther Blotting and hybridization**

An aliquote of 20μl of total RNA in a buffer containing 1X MOPS, 50% formamide and 6.5% formaldehyde was denatured at 65°C for 15 minutes. An appropriate amount of the loading dye (30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol, 1mM EDTA) was then added to the samples and the samples were loaded on 1% agarose gel containing 2.2M formaldehyde. After electrophoresis, formaldehyde was removed from the gel by washing thoroughly in double distilled water with several changes. The gel was stained with ethedium bromide and equal amount of loading was ascertained. The RNA in the gel was transferred to a nylon membrane using the capillary blot method. After blotting, the blot was washed in 2X SSC, air-dried and the locations of the 28 and 18s RNA were marked on the blot. The RNA on the blot was cross-linked using a UV cross linker by irradiating the blot for 120mJoules. The blots were pre-hybridized in 0.5M phosphate buffer pH and 7% SDS at 60°C for for 2 hours. 10⁶ cpm/ml of denatured radioactive probe was added to the same, but fresh prehybridization buffer and incubated for 12-16 hour at 45- 60°C. The blots were then washed and placed in a phosphor imager cassette and the image was read in phospor imager from Fuji BAS 1800 using Fuji image reader software.
Restriction enzyme digestion

Restriction digestion of DNA was carried out with 2-5 units /μg of DNA in 25 to 100 μl of reaction volumes, using the buffers and incubation conditions recommended by the suppliers. The reactions were stopped by raising the reaction temperature to 85/65°C for 10-15 minutes.

Kinase reaction

As 5' ends of primers used for PCR were not phosphorylated, amplified products had to be kinased before these could be used for ligation with dephosphorylated vector. About 10 units of T4 PNK were used for phosphorylation of approx 500 ng DNA in T4 PNK buffer (50mM Tris-HCl, pH 7.7, 10mM MgCl₂, 5mM DTT, 0.1mM spermidine hydrochloride, 0.1 mM EDTA) containing 10mM ATP pH 7.0 in a total volume of 30 μl. Samples were incubated at 37°C for 40 minutes and reaction was stopped by heating at 65°C for 15 minutes. Vector and ligase were added to the same mix and the ligation reaction was set.

Dephosphorylation of the vector DNA

50 ng of Digested and purified vector DNA was incubated with 1 unit of SAP (Shrimp Alkaline Phosphatase) along with 1X SAP buffer (0.5M tris HCl, 50mM MgCl₂ pH 8.5) in a 20μl reaction volume at 37°C for 10 minutes. The reaction was stopped by heating the reaction mix at 65°C for 15 minutes.

Elution of DNA from agarose gel

The portion of the agarose gel containing the DNA fragment was cut out with minimal exposure to UV and equal volume of TE, pH 8.0 was added (w/v) followed by an equal volume of Tris saturated phenol and CHCl₃:IAA (24:1). The entire mix was vortexed vigorously for 2 minutes, frozen at -70°C for 15 minutes and centrifuged at 12K for 15
minutes at 4°C. The aqueous layer was collected again and chilled at -70°C for 15 minutes and centrifuged at 12K for 15 minutes at 4°C to remove the remaining agarose. The supernatant was collected and the DNA was precipitated by adding 4.0μl of sodium acetate and 1ml of ethyl alcohol at -70°C for 1 hour. It was centrifuged at 12K for 15 minutes at 4°C. The pellet was washed with 70% ethanol, dried and dissolved in 10 μl of TE, pH 8.0.

Ligation
Ligation was carried out at 22°C for 5hr to overnight in a 10μl reaction volume containing ligation buffer (50mM Tris-HCl pH 7.6, 10mM MgCl₂, 10mM DTT and 5mM ATP), 50ng of the desired vector, 3M excess of insert DNA and 30 Units of T4 DNA ligase. Recombinant clones were selected by doing colony hybridization.

Colony Hybridization
Colonies were streaked in duplicate on LB plates containing appropriate antibiotic and grown overnight at 37°C. Plates were incubated in the cold room for 30 minutes prior to blotting. Three filter papers were kept on a glass plate and soaked in blotting solutions. Air bubbles were removed by rolling a clean glass pipette on the blotting pads. The transfer membrane was labeled and placed on the plate containing colonies of interest for approximately 1 minute. During the one-minute incubation, the membrane was marked asymmetrically along with the LB plate with a needle containing Indian ink. The membrane was removed carefully and placed face up on the filter paper soaked in denaturing solution for 7 minutes. In the same manner, the membrane was transferred to the filter paper soaked in neutralizing solution for 6 minutes. The filter paper was washed with 2X SSC and the membrane was placed, DNA side up, on the third filter paper soaked
in 0.2M NaOH Solution for 20-30 minutes. The membrane was washed with 5X SSC and processed further for hybridization as described for Southern blot.

**Rapid Amplification of c-DNA Ends (RACE)**

RACE was done according the instructions given in the manual provided with the Clontech RACE kit. Clontech 5' RACE is based on the principle that certain RT enzymes add a stretch of 3-5 cytosine nucleotides when they reach at the end of the template. SMART oligos provided in the kit contain a stretch of Gs along with specific primer sequence.

**First Strand c-DNA synthesis**

Total RNA was isolated from the cells and first strand synthesis was done as follows:

1µg of total RNA was taken in a PCR tube and 1µl of 10µM gene specific reverse primer and 1µl of 10µM SMART II oligonucleotide was added to the tube. Deionized distilled water was added to make up the reaction volume to 5µl. Contents were mixed and the tube was spun briefly. Tube was incubated at 72°C for 2 minutes and cooled on ice for 2 minutes. It then was spun briefly to pellet the contents. 2µl of 5X first strand synthesis buffer, 1µl of 20mM DTT, 1µl of 50X 10mM dNTP and 200 units of RNAseH-MMLV reverse transcriptase was added to the tube and mixed by pipetting it gently. The tube was incubated at 42°C for 1 hour. Termination of the first strand reaction was achieved by incubating the tube on ice.

**PCR**

2µl of the aliquot was taken per PCR reaction for the next step and the rest of the mix was stored at -20°C. The PCR reaction was set by adding the following components to the tube containing 2µl of first strand c-DNA sample: 80µl of deionized water, 10µl of 10X advantage 2 PCR buffer, 2µl of 50X dNTP mix, 2µl of each PCR primer, 2µl of 50X advantage II polymerase mix. The thermal cycler was pre-heated up to 95°C and PCR was
performed using the following conditions: 95°C for 1 min and 25 cycles of 95°C for 15 seconds, 62°C for 30 seconds, 68°C for 1 minute. Finally a 10 minute extension was given. 30 µl of mix was analyzed on the gel. Initially, a range of 15-30 cycles of PCR was tried to get the specific RACE product without excess of smearing.

**Cell Lysate preparation**

Adherent cells were washed twice with ice cold PBS, cells were pelleted at 1K for 1–2 minutes and PBS was removed completely. Cell lysis Buffer (TritonX100 1%, Sodium deoxycholate 1%, SDS, 0.1%, NaCl 150mM, Tris-HCl (pH 7.2) 10mM, Sodium orthovanadate 1mM, inhibitors (added just before use), PMSF 1μl, Apoprotenin 2µg/ml, Leupeptin 2 μg/ml) was added to the flask/dish (1ml/100mm dish or 150cm² flask or 0.5 ml per 60mm dish/ 75cm² flask and it was incubated on ice for 20 minutes to lyse the cells properly. Cells were scraped using a rubber policeman and transferred to the eppendorf tube, whenever required, the cells were sonicated for 5 seconds. The cells were spun at 14,000xg for 5 min and the supernatant was transferred to a fresh tube.

**Quantitation of proteins**

Quantitation of protein was done using Bradford’s reagent obtained from Bio-Rad. Double distilled water was added to equal volumes of protein samples of unknown concentration to make up the final volume to 200µl. 50µl of reagent was added and mixed well. The colour was quantified using an ELISA reader recording the OD at 590 nm. The concentration of proteins was calculated using a standard curve generated using the same protocol and reagents but with known concentrations of Bovine Serum Albumin (BSA).
SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS polyacrylamide gel electrophoresis was carried out by the method described by Laemmli (1970) using a discontinuous buffer system. A solution containing 29% acrylamide and 1% N, N'- methyl bis acrylamide (w/v) was used. The resolving gel had 10% or 8% acrylamide and the stacking gel had 4.5% acrylamide. The gel and the buffers contain 0.1 % SDS. The gels were polymerized using TEMED (10µl for 15ml gel mixture) and 50µl of 20% APS. The gels were cast on a vertical mighty small protein gel apparatus (Hoefer, CA, USA). Prior to loading, the protein samples were heated in a boiling water bath for 5-10 minutes in SDS protein loading dye (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue and 5% β mercaptoethanol). Electrophoresis was carried out in a constant current mode at 20mA till the samples crossed the stacking gel and then at 40mA through the resolving gel.

Staining with Coomassie brilliant blue

The gels were stained with 0.2% Coomassie brilliant blue R250 in methanol:Acetic acid:water (45:10: 45 v/v). The gels were de-stained with the same solution without dye for 6 hours on the rocking platform with 2-3 changes.

Immunoblotting (Towbin et al., 1979)

The protein samples resolved on the SDS-PAGE were transferred to the nitrocellulose membrane by semi-dry graphite method as described in Amersham semi-dry protein transfer apparatus instruction manual:

The gel containing the protein, the C Extra Nitro cellulose membrane and 6 sheets of Whatmann 3M paper cut to the size of gel were soaked in transfer buffer (39mM Glycine, 48mM Tris, 0.037% SDS and 20% methanol) and arranged as follows:
3 pieces of soaked paper were placed on the anode plate. The membrane was placed over it. The gel was aligned on the membrane and 3 more pieces of blotting paper were placed over it. Care was taken to remove air bubbles from the 3M sheets as they interfere with the transfer. The transfer was performed for 2 hrs using a current of 0.8-mA/cm². The efficiency of transfer was subsequently checked by staining the membrane with Ponceau stain.

The membrane containing transferred protein was incubated with TBS containing 3%BSA for 1 hour. This procedure, called blocking, is used to block the free spaces on the membrane with a neutral protein - BSA. The blot was rinsed once with TBS, incubated with the recommended dilution of primary antibody in TBS containing 1%BSA for 1-2 hours. The blot was washed with TBST (0.1% Tween-20) for 30 minutes with 3 changes. The blot was incubated with either 1:10,000 dilution (HRP tagged) or 1:1000 dilution (ALP tagged) of secondary antibody in TBS with 1% BSA for 45 minutes -1 hour. It was then washed with TBST for 30-60 minutes with 4-5 changes of solution on a rocking platform.

For ECL method (HRP tagged Secondary antibody), the blot was removed from the wash solution and placed within a polythene cover; developing solution mix (Roche) was spread on it using a pipette and incubated for one minute. Excess solution was removed from the blot and the blot within the polythene was exposed to an x-ray film that was subsequently developed.

In the chromogenic method (ALP tagged Sec Ab), the blot was placed in ALP buffer containing NBT/BCIP (substrates for Alkaline Phosphatase) and kept on a rocker till clear bands appeared on the blot. The blot was rinsed with TBST and dried using blotting paper.
Protein expression

The gene of interest was cloned in pET21a vector and sequenced for confirmation of correct reading frame. BL21 competent cells were transformed with recombinant plasmid and grown on LB plate containing 100μg/ml ampicillin. A single colony was inoculated in 10ml of LB medium containing 100μg/ml ampicillin and grown overnight in a 50ml flask at 37°C. 100ml of pre-warmed media containing antibiotic was inoculated with 5ml culture and grown at 37°C with vigorous shaking till OD₆₀₀ reached 0.6. Prior to induction a 1ml aliquot of culture was taken; cells were pelleted and suspended in 60μl of 4X SDS PAGE sample buffer. Expression of recombinant protein was induced by adding IPTG to a final concentration of 1mM. The culture was grown for another 12 hours and samples were collected at 2hr, 4hr, 8hr, 12 hr; cells were pelleted and re-suspended in 120μl of SDS-PAGE sample buffer. SDS-PAGE analysis was done using induced and uninduced samples to determine optimum expression time.

Protein purification under native conditions

Protein was induced as described earlier, cells were pelleted at 4000Xg for 20 minutes and the cell pellet was stored at -20°C overnight. Cell pellet was thawed on ice for 15 minutes and the cells were re-suspended in 4ml of lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH8.0, adjusted using NaOH). 1mg/ml lysozyme was added in the lysate and incubated on ice for 30 minutes. Cells were sonicated using six 10-second bursts at 200-300W with a 10-second cooling period between each burst. The lysate was centrifuged at 10,000xg for 20-30 minutes to pellet the cellular debris and the supernatant was collected. 1ml of 50% Ni-NTA slurry and 4ml-cleared lysate were mixed gently by shaking at 200rpm on a rotary shaker at 4°C for 60 minutes. Lysate Ni-NTA slurry was loaded into a column and the excess fluid was allowed to flow through. The column was
washed twice with 4ml wash buffer (50mM NaH2PO4, 300mMNaCl, 20mM imidazole, pH8.0, adjusted using NaOH) and the protein was eluted 4 times using 0.5ml elution buffer (50mM NaH2PO4, 300mMNaCl, 250mM imidazole, pH8.0, adjusted using NaOH).

Protein purification under denaturing condition
The cell pellet stored at -20°C was thawed for 15 minutes on ice and resuspended in bufferB (100mM NaH2PO4, 10mM Tris-HCl, 8M urea, pH8.0, adjusted using NaOH) containing 8M urea (5ml per gram wet weight of cells). Cells were lysed by gentle vortexing till the solution became translucent. Further processing was carried out as described above. In this case, however, different washing buffer (100mM NaH2PO4, 10mM Tris-HCl, 8M urea, pH6.3, adjusted using HCl) and elution buffer (100mM NaH2PO4, 10mM Tris-HCl, 8M urea, pH5.9 adjusted using HCl) were used.

Generation of polyclonal antibody
Collection of control sera from animal
Blood collected from the animal was incubated at 37°C for 1 hr and at 4°C for 2-3 hr, Serum (supernatant) was collected by centrifuging the sample at 2500- 3000 rpm for 30 minutes. The serum was stored at -70°C in 100-150μl aliquots.

Polyclonal Antibody
For raising polyclonal antibody against a Cullin 4B protein in the rabbit, 100μg of protein was mixed with 500μl of Freund’s complete adjuvant and volume was made up to 1ml with PBS. The mixture was made into an emulsion by passing through a 2ml syringe (1.5inch 19G needle) and intermittently keeping it at -20°C. This emulsion was injected into the rabbit subcutaneously as well as intra-muscularly. After 20 days, the first booster dose was given in a similar manner, except that Freund’s complete adjuvant was replaced with Freund’s incomplete adjuvant. Subsequent booster doses were given after a 15-day
interval and the antibody titer was checked using dot blot. For determination of antibody titer 20ng protein was spotted in a row, air-dried and incubated with different concentrations of immune serum (1-100 to 1-50,000). Western blot experiments were carried out when the appropriate titer (minimum of 1:10,000) was obtained following booster doses. Purification of antibody specific to the protein of interest was carried out using immuno-affinity method to circumvent background signals in the western blot.

**Affinity purification of polyclonal antibodies**

Elution of antibody was done following the method of Talian et al. (1983). The protein gel was run and a immuno-blot was carried out as described above; the blot was stained with Ponceau S. Antigen and the strip of interest was cut from the blot, and blocked with 3% BSA for 1 hour at room temperature. It was then incubated with primary antibody (1:200 in blocking buffer, sodium azide to 0.02%) overnight at room temperature on a rocker with gentle agitation. The blot was washed twice, for three minutes each with 0.1% TBST. For antibody elution 5ml of 100mM glycine, pH 2.6, was added to the blot for 2 minutes. 5ml of glycine buffer containing antibody was removed carefully and 1M Tris, pH 8.5, was added to a final concentration of 50mM for neutralization of antibody solution. Finally 30μl of NaN₃ was added to avoid contamination before storing the antibody at 4°C. Antibody purified this way is mono specific polyclonal antibody. 1%BSA (final concentration) in TBS containing 0.02% azide was added to the antibody solution for stability. This solution was used as primary antibody in western blots.

**Co-immunoprecipitation** (Katsutoshi et al, 1998, Piefer et al, 1990)

Cells / Drosophila embryos were washed twice with PBS and PBS was removed completely. Ice cold modified RIPA (For 100 ml, 50mM Tris-HCl, 5ml 1M Tris (pH 7.4),
150mM NaCl 0.8775g, 2mM NaF, 1mM EDTA, 1mM EGTA, 1mM NaVo₃, 1% TritonX 100, PMSF 1mM, Apoprotein (20μg/ml), Leupeptin (20μg/ml) buffer was added to the cells (1ml/10⁷ cells per 100 mm dish or 150 cm² flask; 0.5ml/5x10⁶ cells per 60mm dish or 75cm² flask) and either (as the case may be) the cells were scraped using a cooled rubber police man or the embryos were homogenized in an eppendorf tube using a homogenizer. The embryo or cell suspension was transferred to the centrifuge and mixed gently on a rocker for 15 minutes to lyse the cells completely. Lysate was centrifuged for 15 minutes at 14,000xg and the supernatant was transferred immediately to a fresh tube.

ProteinG-Sepharose was prepared by washing the beads twice with PBS and resuspending them in 50% PBS. The lysate was pre-cleared by adding 100μl of beads per ml of lysate, and incubating them for 1h on an orbital shaker at 4 °C. The mixture was spun at 14,000xg for 10 minutes and supernatant was collected in a fresh tube. The protein concentration was determined and the lysate was diluted to yield a final protein concentration of 5mg/ml. Affinity purified Cul4 antibody was added at a final dilution of 1:50, to the cell lysate. The antibody-cell lysate mix was incubated at 4°C on an orbital shaker overnight.

On the following day, 100μl of proteinA-Sepharose beads were added to the lysate-antibody mixture and incubated for 2 hours at 4°C on orbital shaker. To recover the immuno-complex, the beads were collected by 5 seconds pulse centrifugations at 14,000 rpm. The supernatant was discarded and the beads were washed thrice with modified RIPA buffer containing detergent, by incubating them with the buffer for 5 min each on the orbital shaker at 4°C. The beads were recovered by pulse centrifugation, resuspended in 60μl of sample buffer and boiled for 5 minutes to dissociate the complex from the beads. The samples prepared were used further for the immuno-blot analysis.
**Cell fractionation** (Judith et al. 2002)

Cells were fractionated into nuclear and cytoplasmic fractions by lysis in an NP-40-based buffer (10mM Tris–HCl, pH 7.5, 10mM NaCl, 3mM MgCl2, 0.5% Nonidet P-40, pH 7.5, supplemented with protease inhibitors (1mM N-([aminoethyl) benzenesulfonylfluoride, 40μM bestatin, 15μM E64, 20μM leupeptin, 15μM pepstatin; Sigma). Following incubation on ice for 10 minutes, the lysates were centrifuged at 15,000g for 10 minutes. The supernatant (cytoplasmic fraction) was collected and stored; the pellet (nuclear fraction) was washed by re-suspension in a fresh aliquot of lysis buffer and centrifugated as mentioned above. The pellet from this second centrifugation was then suspended in RIPA buffer, sonicated, and centrifuged to remove insoluble debris, and the supernatant was stored. The quality of fractionation was verified by probing blots prepared from cellular fractions with anti-SOS antibodies; SOS was detected exclusively in the non-nuclear fractions.

**Whole mount In Situ hybridization**

Whole mount in situ hybridization was carried out as described by Linda A. Lowe and Michael R. Kuehn.

**The protocol is divided into 3 parts:**

- **Embryo dissection**
- **Ribo-probe preparation**
- **Wholemount in situ hybridization**
- **Embryo dissection**

Embryos were dissected in PCM. All the embryos were collected in an organ culture dish containing PCMF, on ice till dissection was completed. After all the embryos were dissected, they were transferred to one or two baskets using a cut tip. The embryos were
then rinsed with PBT. For each basket, two rows of 24-well plates were set (12 successive wells).

**Ribo-probe Preparation**

A restriction enzyme reaction was setup to linearize the plasmid DNA; complete digestion was confirmed by a gel run and it was then incubated at 80°C for 20min. The digested plasmid was recovered by ethanol precipitation. The plasmid DNA pellet was washed with 80% ethanol. It was subsequently re-dissolved to a concentration of 1mg/ml in RNase free TE buffer. The labeling reaction consisting of 1μg of prepared plasmid DNA, 2μl of 10X DIG RNA labeling mixture, 2μl of 10X transcription buffer, 5μl of 100mM DTT and 2μl of T7 RNA polymerase was set up. RNase-free water was used to make the volume up to a total of 20μl. The last 4 components of the reaction mixture were obtained from an Epicenter technologies T7 transcription kit. The reaction mixture was incubated at 37°C for 2hours. 1μl of 10X DIG RNA labeling mix, 1μl of 10 X transcription buffer, 2.5μl of 100mM DTT, 1μl of polymerase and 4.5μl of RNA free water were added. The reaction mixture was further incubated at 37°C for 1hour. 1.5μl of 0.4M EDTA and 3.75μl of 4M LiCl were added, and the contents were mixed. 112.5μl of 100% ethanol was added and the mixture was incubated at -20°C overnight. Labeled RNA was pelleted by centrifuging at top speed in a microfuge. The pellet was washed with 80% ethanol and dissolved in 110μl of RNase-free water. It was then re-precipitated using 12.5μl of 4M LiCl and 250μl of 100% ethanol at -20°C. This was centrifuged once more at top speed and the pellet was washed with 80% ethanol. Labeled RNA was dissolved in 25μl of RNase-free water and 1μl of it was examined on a 1% agarose gel.
Whole mount *In situ* hybridization day 1

**Probe hybridization**

Twelve successive wells of 24 well plates were filled with the following solutions for each basket of embryos to be hybridized. 75% Methanol in PBT in one well, 50% Methanol in PBT in another well, 25% Methanol in PBT in one well, PBT alone in two wells, Proteinase K(PK) at final concentration in two wells, PBT in one well, PGF for post fixation in one well, PBT in two wells, 1:1 PBT/hybridization buffer in one well and hybridization buffer in one well. The embryos were re-hydrated by taking the basket through the wells with methanol in PBT (5 minutes each). They were then washed and rinsed with PBT. The basket was put into the PK well and incubated for an empirically determined time. The basket was removed from PK, quickly rinsed with PBT and put into PGF to stop the PK reaction. The embryos were post-fixed for 20 minutes, rinsed and washed with PBT. They were prepared for hybridization by rinsing with PBT or hybridization buffer. The embryos were allowed to settle before proceeding. The basket was removed from the final well of the 24-well plate and placed in a Bijou tube with 1 ml hybridization buffer. The vial was capped and placed at 65°C for pre-hybridization for 1 hour. An additional 1ml of hybridization buffer was added to Bijou (pre-warmed at 65°C) containing 1µl DIG-labeled ribo-probe, placed immediately at 70°C and hybridized overnight.

Whole mount *In situ* hybridization day 2

**Antibody Reaction**

The probe solution was removed following overnight hybridization. The embryos were rinsed twice with 2ml of hybridization buffer (pre-warmed at 65°C). They were washed twice for 30 minutes each with 2ml of hybridization buffer pre-warmed to 55°C. For each basket of embryos, five successive wells of a 24-well plate were filled with 2ml of the
following solutions - three wells with TBST, one well with blocking buffer A, one well with blocking buffer B. The basket was removed from the Bijou vial and put through the first two wells with TBST to rinse the embryos twice. It was then placed in the third well with TBST to wash the embryos for 15 minutes. The basket was transferred to blocking buffer A and incubated for 1 hour with gentle rocking. It was then shifted to blocking buffer B and incubated for another 1 hour with gentle rocking. AP coupled Anti-DIG antibody (1:2000 dilution) was made by diluting 1μl in 2ml of blocking buffer B (pre-chilled on ice) in a Bijou vial. The basket was transferred to the Bijou vial, capped, and incubated at 4°C overnight with gentle rocking.

Whole mount In situ hybridization day 3
Detection
Following the overnight antibody reaction, the basket was removed, washed with 10ml TBST thrice for 1 hour each, with gentle rocking. The embryos were removed from the basket and transferred to an organ culture dish using a plastic transfer pipette (cut-enlarged the opening for older embryos). Carried over TBST was removed using a transfer pipette. Embryos were rinsed with water and 0.5ml of BM purple AP substrate was added to the plate which was incubated at room temperature. The staining reaction was terminated when sufficient color was accumulated by carefully removing the AP substrate solution and adding 1 ml of PBT. Fresh PBT was added to the embryos and washed twice for 5 minutes each.

Materials for RNA In Situ hybridization
1. Dissection tools
   Curved shank Dumont forceps and fine angled scissors, 2 Dumont 5/4 MC INOX forceps with 45° angled tips, 2 Dumont 5 INOX forceps with Biologie tips.
2. Baskets

Polypropylene tube (2ml), nylon mesh (100-200μm). Baskets were made by removing the conical bottom of polypropylene tubes and attaching the nylon mesh.

3. Sterile plastic ware

Sterile plastic transfer pipettes, 7ml Bijou tubes, 24-well cell culture plates, organ culture dishes.

4. Embryo dissection and wash solution

PBS without CaCl2 and MgCl2, PCM: PBS with CaCl2 and MgCl2, PCMF: PCM supplemented with 10% FCS, PBT: PBS without CaCl2 and MgCl2 supplemented with 0.1% Tween-20.

5. Solution for fixing embryos

4% Para formaldehyde in PBS, prepared by heating at 60-65 °C with stirring.

PGF: Para formaldehyde / gluteraldehyde - 4% Para formaldehyde, 0.025% Gluteraldehyde in PBS.

6. Solutions for dehydrating and rehydrating Embryos

25%, 50% and 75% Methanol in PBT, 25%, 50 % and 75% methanol in water, 100% methanol.

7. Solutions for Proteinasing Embryos

PK stock solution: 100μg/ml Proteinase K solution

Working solution: 5, 10, 20μg/ml in PBT.

8. Ribo-probe preparation

T7, SP6 and T3 in vitro transcription kits (Epicenter Technologies), 10X DIG labeling mix (Boehringer Mannheim), 80%, 100% ethanol, 5M NaCl, TE buffer, 0.4M EDTA and 4M LiCl.

9. Hybridization solutions
1M PIPES (Sigma), 10X PE, 100mM PIPES and 10mM EDTA, Hybridization buffer: 50% Formamide, 0.75M NaCl, 1X PE, 100μg/ml tRNA (Sigma 10mg/ml), 0.1% BSA, 1% SDS, 0.1% Tween-20 in DEPC water prepared fresh each time.

10. Antibody detection

1. Solution

10X TBS (8g NaCl, 0.2g KCl, 0.2M Tris-HCl, pH 7.6 made up to 100ml) stored at 4°C. TBST: 1X TBS and 0.1% Tween 20 made fresh each time. Blocking reagent stock: 10% suspension of Boehienger blocking reagent (BBR) in 1X TBS prepared by heating at 70°C for 30 minutes. Aliquoted 10ml and stored at -20°C. Goat serum (Sigma) heat inactivated at 70°C for 30 minutes, Blocking buffer A: with 2% BBR, Blocking buffer B: with 2% BBR and 20% goat serum. AP inactivation buffer: 100mM lysine, pH 2.2, and 0.1% Tween-20.

2. Antibodies

AP Coupled DIG antibody, Fab fragment (BM)

3. AP substrate

BM pupple AP substrate
## Primers, Constructs And Sequences

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<td>EcoRV</td>
</tr>
<tr>
<td>CBN</td>
<td>pMOS</td>
<td>EcoRV</td>
</tr>
<tr>
<td>CBN</td>
<td>pET21a</td>
<td>BamHI and XhoI</td>
</tr>
<tr>
<td>N- terminus</td>
<td>pEGFPN1</td>
<td>BamHI</td>
</tr>
<tr>
<td>CBN</td>
<td>pBSKS</td>
<td>SmaI</td>
</tr>
<tr>
<td>CBC</td>
<td>pMOS</td>
<td>EcoRV</td>
</tr>
<tr>
<td>CBC</td>
<td>pET21a</td>
<td>Nhel and XhoI</td>
</tr>
<tr>
<td>CBC</td>
<td>pEGFPC1</td>
<td>XhoI and SmaI</td>
</tr>
<tr>
<td>dCul4</td>
<td>pBSKS</td>
<td>SmaI</td>
</tr>
<tr>
<td>dCul4</td>
<td>pUAST</td>
<td>KpnI and NotI</td>
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Sequences

*Mus musculus* Cul4B protein SEQUENCE (970 a.a)

MSRSTRSKERRENDTSEDNSSETSNQERRRRCRQGGPPRPYPPPLPPVFPPPTPPQV
VRTRGLQDLGAMSKCPGTSFSSPNPSAASAAAQEVRSATDGNSTTTTPPSAK
KRKLNSSSSSSSNENEDFDSTSSSSTPQPRDASASPSTSSFCGLVPATSSSHVPIQ
KKLRFEDTLEFVGIDTKEAEESSSSSSSPPAAATSSQQQQQQLKTSAISVASH
HANGLAKSSTAVSSFANSPKPSAKLVIKNFKDKPCKPENYTEDTWQKLKEAV
EAIQNSTIKYENLELYQAVENLCSHKISANLYKQLRQICEDHIKAQIHQFBREDLD
SVLFLKIDRCWQNHCRQHMIMIRSLFLDLRTVYLNQSNMLPSIDMGELFRHII
SDQKVQTGDIGDILLNREIDSSRLSLSSMLDSLQIYDQSFQFQLPETN
RLYAAEGQKLQMRPSPEVPEYLVHVNKRLEEEADRLVLDQTDQQKSLIASVEKQL
GEHLTAILQKGLNSLDENRQDLQYLLLYQLSRVRGQVLLQFQIYEUKAFSTI
VINPEKDFTMVQELLDFKDVKHDICTFCLNKEFIMAKEFETFINKRPNKAE
LAIYVDSDKLRAGNEATDEEELMDKMIIFRIFYKDVEAFYKDLAKRL
VGKSASVDAEKSMKSLKHECGAAFTKLEGMFKDMELSKEIMFKQYMQNQ
NYPNQNELTNMLTGMYWPTYQPMVHLPEMVLQIEIFKNFYLKSHGRKLQW
QSTLQHCVLKAKEFKEKQKSLQFQTMVLLMFNEGEFEEFLEIEEKHTAGIED
RRTLQSLACGKARVLANKPKGKDIEGDKFIICNDDFKHKLFKRIKINIQMQKETVE
EQASTTERVFQDRQYQIAIVRMIMKMKRLTSHNLVSEVYNRLKFPVKPADLK
KRIEISLIDRYMIEDKERNNPYNYIA

Note: Nuclear localization signal

*Mus musculus* Cul4A protein sequence (759 a.a)

MADEGPGRKSGVSALMGRTNGLTJKPAAALAGGPKPGTGGRSKLVIKNFRDPRPL
PDNYTDQTDWRKLHEAVKAIQSSTSIRYENLELYQAVENLCSHKVSTPLYKLQROV
CEDHVQAIQLPREDLSVFLLKINTCWQHDRCQHMIMIRSLFLDLRTVYLNQNSSMLPSIDWMGELFRHRSSMRVQSKTIDGIIAGREGSEAVDRSSLRLSSMLS
DLQYKDSFLKFLFETYTCPNLYAAEGQRLMQDREVPEYLVHNSKREEEADRVIT
YLHDHSQKPLACVEQLQLGEHLTAILQKGLHEHLDDNEPDWLQMYQLSFRKG
GGHALLQHISEYIKTFGTINQPKEDKMDVQDLLDFKDVKHDVHEVCFQRNRF
FNLMKESFETFINKRPNQELAIKHDVSLRAGNEATDEEELMDKIMIRFHI
HGKDVFSEAFYKDLAKRLVKGKASAEDAEKSMKSLKHECGAATASKLEGMF
DMELSKEIMFKQHMQNSSAPGPDILTVNTMLTGMYWPTYQPMVHLPEMVR
LQEVKFTFYLKSHGRKLQWQTTLGHAVLKDADFKEGGKKEFQVSLFTQLLLMNF
EGDGFSEDFKMATDSLRRQTSQSAUCGKARLIFSKPGKEEDGKDFFINAD
FKHKLFRKIKINIQMQKETVEEQSVSTTERVFQDRQYQIAIVRMIMKMKRLTSHNL
LVSELYNLKFPVKPDGLKKRIEISLIDRYMIEDKERNPQNYHYVA

51
Mus musculus cul 4B Nucleic acid sequence

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gagttcgttgccgggccagtcgaagcagacaagctcttcagaaggaagttgctgcatgatctgatcttgatggtcagcgtcat
tgctcaactaggctctaaaggaaggagagaagagtcgaagctcattggaagatctgctgcagctgaccctcttttaaacctt
gcggaggaaggtgcgcaacggcctcttttcaacccctgcttctccctccttccctacccctgtcagcttcggcaccggggtttc
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