

# Cloning and characterization of mouse cullin4B/E3 ubiquitin ligase

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Heat induced differentiation of mouse embryonal carcinoma cells PCC4 has been reported earlier. We have further characterized the phenotype of the differentiated cells and by DD-RT-PCR identified several partial cDNAs that are differentially expressed during differentiation. Nucleotide homology search revealed that the genes corresponding to some of the up-regulated partial cDNAs are indeed part of differentiation pathway. 5' extension of an EST that has homology to one of the partial cDNAs led to the identification of mouse cullin4B. Cullin4B is coded by a separate gene and has a unique and longer amino-terminal end with a putative nuclear localization signal sequence (NLS). We have cloned, expressed and raised antibodies against the amino and carboxy-terminal halves of cullin4B. Immuno staining of differentiated PCC4 cells with N-terminal Cul4B antibody showed enhanced expression of Cul4B and its translocation into the nucleus upon differentiation. Transient transfection of a chimeric gene encoding the N-terminal part of Cul4B fused to green fluorescent protein into PCC4 cells revealed that the protein was localized in the nucleus confirming the functional significance of the putative NLS. Since cullins are involved in recognition of specific proteins for degradation, based on the evidence presented here, we hypothesize that cullin4B is probably involved in differentiation specific degradation/modification of nuclear proteins.

[Tripathi R, Sastry K S, Kota S K and Srinivas U K 2005 Cloning and characterization of mouse cullin4B/E3 ubiquitin ligase; *J. Biosci.* **30** 329–337]

## 1. Introduction

During early embryonic development the complex pattern of cell types and structures arises through a process of differentiation wherein pluripotent, unspecialized cells take on individual characteristics and reach their mature, specialized form and function. The cellular events that lead to the commitment of cells to new phenotypes and the changes in the expression pattern of genes (Nothias *et al* 1995) is key to understanding development. In the embryo, cells of the inner cell mass (ICM) give rise to all embryonic and extra embryonic tissues (Strickland and Mahdavi 1978). Embryonal carcinoma (EC) cells, derived from the inner cell mass of the early embryos are proven as a useful model system (Hogan *et al* 1983; Belloch *et al* 2004) to study the different aspects of development

and differentiation. Change in gene expression that occur during differentiation result in activation or up regulation of an array of genes directly involved in differentiation (Harrison *et al* 1995; Stuart *et al* 1995) and down regulation of proliferation specific genes (Kamitani *et al* 1997; Tateishi *et al* 2001) as proliferation and differentiation often represent mutually exclusive pathways and cessation of proliferation is associated with the onset of differentiation (Sherr 1994). Depending upon the trigger or inducer, EC cells differentiate into a variety of lineages (Strickland *et al* 1980; Sleight 1985; Bisht *et al* 1994; Bath *et al* 2001). In addition to lineage specific genes (Vidricaire *et al* 1994; Braun and Arnold 1996; Lako *et al* 2001), a number of other genes are also activated during differentiation like kinesin like protein, caspase, rho, rac, p27 etc. (Rodriguez *et al* 1997; Ruden *et al*

**Keywords.** Cul4B; DD-RT-PCR; differential expression; differentiation; nuclear localization sequence; PCC4 cells; translocation

Abbreviations used: EC, Embryonal carcinoma; NLS, nuclear localization signal sequence; ORF, open reading frame; TPA, tissue plasminogen activator.

1997; Baldassarre *et al* 1999; Quinlan 1999; Fernando *et al* 1999) that help the cells to modulate their function and in establishing differentiated phenotype.

Aim of the present study is to understand early embryonic development and identify genes, expressed during this process using mouse embryonal carcinoma cells PCC4. Differentiation of EC cells upon heat shock (Bisht *et al* 1994; Maruyama *et al* 1996) and upon curcumin treatment (Batth *et al* 2001) has been reported earlier. In the present study we have further characterized the differentiated phenotype of PCC4 cells, and using DD-RT-PCR, identified several genes that are differentially expressed. In addition we have characterized cullin4B, a gene upregulated during the differentiation of PCC4 cells.

Mammalian cullins are a family of 7 proteins identified so far (Latres *et al* 1999). Cullins are part of E3 ubiquitin ligase complex, acting as scaffold protein which helps in getting the F-box/F-box like proteins (recognizes substrate for polyubiquitination) in the vicinity of E2 enzyme (carries polyubiquitin chain) (Latres *et al* 1999), finally helping in transfer of ubiquitin chain from E2 to the substrate (Cenciarelli *et al* 1999; Winston *et al* 1999; Wilkinson 2000). Transition of cells from pluripotent to mature differentiated stage requires several molecular and biochemical changes. Elimination of several proteins associated with the maintenance of pluripotent state of the cells is one of the important processes, which requires up regulation of proteasome pathway (Cheng-Yao *et al* 2002). Up regulation of Cul4B may be a consequence of this process hence Cul4B was characterized further.

## 2. Materials and methods

Cell-culture, molecular cloning and associated nucleic acid and protein techniques were as per the standard procedures. Anti-lamin A (gift from Dr Veena K Parnaik, CCMB, Hyderabad), anti-actin (Sigma, St. Louis, USA) monoclonal antibodies were used in this study. FITC-labelled actin-phalloidin was purchased from Molecular Probes, USA.

Maintenance and heat-shock induced differentiation of PCC4 cells was as described earlier (Bisht *et al* 1994; Batth *et al* 2001). Total RNA (Chomezynski and Nicoletti 1986) and/or proteins were isolated from cells differentiating for 48 h, unless mentioned otherwise. Differential-display RT-PCR was as described earlier (Liang and Pardee 1995; Liang *et al* 1994a,b) and in the instruction manual provided by the manufacturer (Gene Hunter). 5'RACE was performed following the procedure given in GIBCO-BRL instruction manual. For RT-PCR analyses of Cul4A and Cul4B transcripts, primers were designed to amplify 3' end of each transcript.

### 2.1 Cloning, expression and generation of antibodies

Open reading frame (ORF) for Cul4B was predicted from EST (AY330868). Primers were designed to amplify amino (aa 70–310) and Carboxy (aa 357–970) terminal of Cul4B, cloned in pMOSblue and sequenced. Proteins were expressed along with 6x His tag and purified by Ni-NTA column (Quiagen, Valencia, USA). Polyclonal antibodies were raised against recombinant C-terminus and N-terminus proteins by injecting the purified protein with Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) into the rabbit. After ascertaining the antibody titer, antibodies were affinity purified by probing the membrane containing recombinant protein with polyclonal antibody. Band corresponding to the recombinant protein was cut and monospecific antibodies were eluted at pH 2.8 with glycine buffer, neutralized and used for Western blot and confocal studies.

### 2.2 Immunofluorescence

PCC4 cells were cultured on glass coverslips for 24 h prior to any treatment. Control or differentiated cells were processed as described earlier (Batth *et al* 2001). Briefly, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.5% Triton X-100 for 10 min. Cover slips were blocked with 5% non-immune serum from the same species as the labelled secondary antibody, incubated with actin phalloidin (Molecular Probes), or lamin A antibody or affinity-purified anti-Cul4B antibody (1 : 100 dilution) in phosphate buffer saline (containing 5% non-immune serum from the same species as the labelled secondary antibody) at room temperature for 1 h. The cells were washed and incubated with FITC conjugated secondary antibody (1 : 200) for 45 min then washed in phosphate buffer saline and mounted in medium containing DAPI (counter stain) and examined by confocal microscopy.

## 3. Results

### 3.1 Characterization of PCC4 cell differentiation

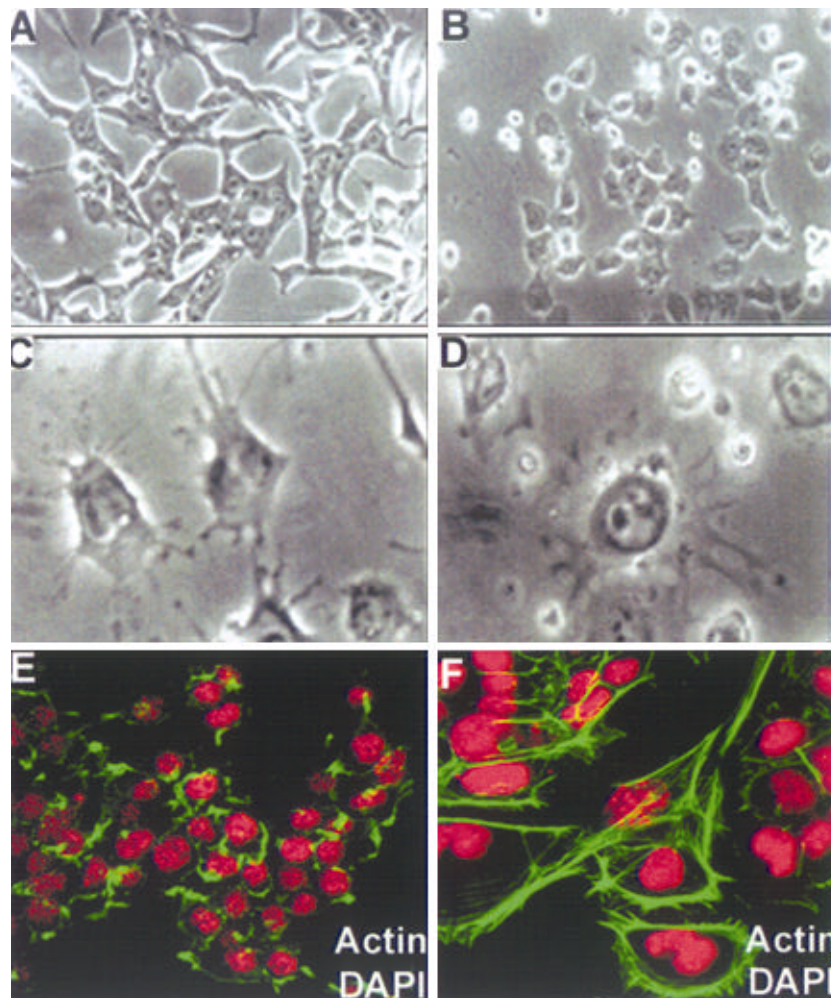
We have reported earlier that the pluripotent PCC4 cells which are usually small with distinct cell boundaries and distinct nuclei (figure 1A) differentiate upon heat shock into large well-spread cells with reduced nuclear to cytoplasmic ratio, and more distinct cell boundaries. As shown in figure 1, though PCC4 cells appear detached even after 6 h of recovery at 37°C after heat treatment (figure 1B), cells were attached and differentiated by 36 h (figure 1C,D). Actin-phalloidin staining showed changes in actin cytoskeleton. Compared to control cells differentiated

cells showed extensive networking of the actin filaments (figure 1E,F). Differentiation of these cells was further confirmed by the expression of differentiation specific markers like lamina A and tissue plasminogen activator (TPA). Lamin A that has been demonstrated to have a differentiation specific expression (Batth *et al* 2001) is also expressed upon differentiation of PCC4 cells (figure 2A,B). Expression of TPA (Casanova and Grabel 1988) detected by Northern blot (figure 2C) confirms differentiation of the PCC4 cells to endodermal lineage. Expression of other lineage specific markers like HCG, keratin and MyoD was negative.

### 3.2 Identification of differentially expressed genes

In a screen to identify genes, whose transcription levels

are modulated during differentiation, differential-display RT-PCR analysis was carried out with RNA from undifferentiated and differentiated PCC4 cells. Several transcripts were identified, which were differentially expressed between control and differentiated PCC4 cells (figure 3A,B). Differential expression of cDNAs was confirmed by the Northern blot analysis (figure 3C). cDNAs that showed clear up or down regulation upon differentiation (figure 3C) were subsequently cloned into pMOSblue vector, sequenced and BLAST search was performed to find out the identity of transcripts. Results of such analysis, presented in table 1 shows the identity of differentially expressed partial cDNA. Of the fragments analysed, cDNA sequence of H1 showed homology to a short region of kinesin-like protein, which is expressed during testis development. Another cDNA, H4, showed homo-



**Figure 1.** (A–D) Differentiation of PCC4 cells. Exponentially growing PCC4 cells were given heat shock for 45 min at 42°C and allowed to recover at 37°C. (A) Control; (B) 6 h; (C) 24 h; (D) 48 h after heat shock. (E) Control PCC4 cells show actin bundles in the peripheral cytoplasm. (F) Upon differentiation well-spread actin fibers are visible.

logy to NAP1-like protein (nucleosome assembly protein 1 like protein), which was known to be expressed during differentiation. cDNA sequence of H3 did not show any homology to any EST, hence this novel gene sequence was submitted to GeneBank (Accession No. BE635344 dbEST Td5866663; used Id PCC4 est.1). cDNA sequence of O2, which showed down regulation during differentiation matched with an EST (BB504873) from neonatal kidney and stem cells.

### 3.3 Cloning and molecular characterization of mouse *cul4B*: Comparison with *cul4A*

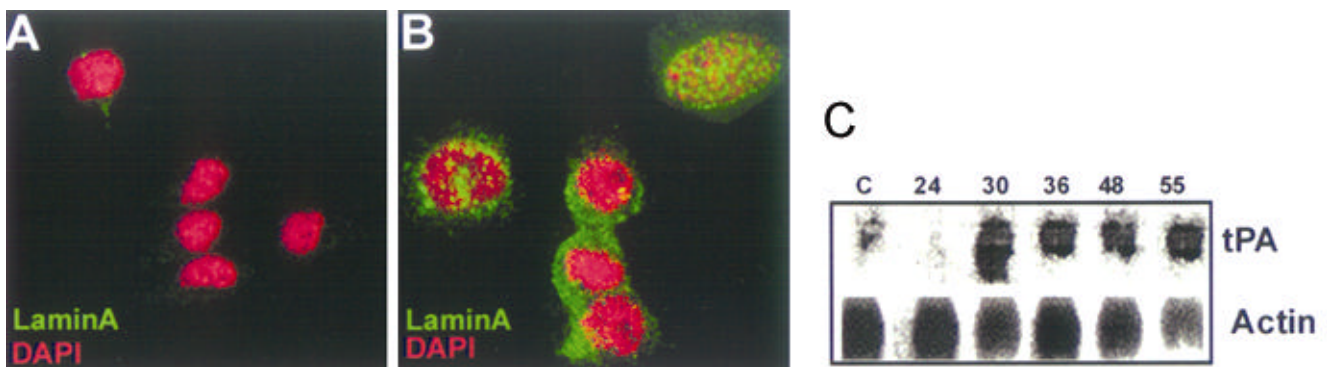
Nucleotide homology search with 320 nt long partial cDNAs (H2) showed homology to an EST representing partial mouse *cul4A*. Based on the EST sequence gene specific primers were designed, 5' prime RACE was done and the 1 kb RACE product was cloned and sequenced. DNA and protein database homology search, done with the sequence obtained by RACE and its ORF identified two proteins, *Cul4A* and *Cul4B*, which were highly homologous at the protein level but relatively less homologous at the DNA level. Full-length *Cul4B* transcript, compiled from RT-PCR and data base searches was submitted to the GENBANK (GI: 32483453). At the genome level, *cul4B* is spread over a 43 kb region on the X chromosome with 23 exons and 22 introns. RT-PCR with the RNA isolated from the control and differentiated PCC4 cells confirmed differential expression of *Cul4B* (figure 3D). As shown in the figure 3D *Cul4B* was up regulated during differentiation and was more abundant compared to *Cul4A* in differentiated PCC4 cells.

In both mouse and human, two closely related proteins represent *Cul4* (*Cul4A* and *Cul4B*), coded by two differ-

ent genes (Li *et al* 2002). Detailed molecular characterization of mouse *Cul4A* has been reported by Li *et al* (2002). Full-length *Cul4A* and *Cul4B* transcripts are 3477 bp and 3346 bp long, respectively. *Cul4A* polypeptide is 759 aa long, whereas *Cul4B* polypeptide is 970 aa long. The main difference between mouse *Cul4A* and *Cul4B* proteins is at the amino terminus; *Cul4B* is longer by 211 amino acids. At the nucleotide level *Cul4A* has a longer 3'UTR. Rest of the sequences show up to 90% similarity (figure 4). However, at the DNA level, the two genes are far less similar, which is attributable to the degeneracy of the third base in nearly 50% of codons. The unique N-terminal domain of *Cul4B* allowed specific characterization of *Cul4B*, which is the main focus of this study.

### 3.4 Expression of recombinant *Cul4B* and generation of specific antibodies

We cloned and expressed unique N terminal and C terminal regions of *Cul4B* in bacteria. Recombinant proteins were purified using Ni-NTA affinity column and authenticity of the two proteins was confirmed by MALDI-ToF analysis. Out of 65 peptides 35 of them corresponded to *Cul4B*. Purified proteins were injected into the rabbit to raise polyclonal antibodies against the two domains. To avoid any background caused by polyclonals, antibodies were affinity-purified to obtained monospecific antibodies (as described in § 2), which were used for further experiments. Western blot analyses of total proteins isolated from PCC4 cells using antibodies against the C-terminus (CBC) detected both *Cul4A* (~ 83 kDa) and *Cul4B* (figure 5A), whereas antibodies against the N-terminus (CBN) detected only *Cul4B* (~ 105 kDa) (figure 5A). CBN antibody cross-reacting with a single band in the total cell lysate confirmed the specificity of the antibody.



**Figure 2.** PCC4 cells differentiate into endodermal cell lineage upon differentiation. (A–B) Lamin staining pattern. Control and differentiated cells were processed for immunostaining with antibody to lamin A. (A) Control cells show complete absence of staining whereas (B) differentiated cells have big nuclei with lamin A appearing as a rim around the nucleus. Magnification 100 $\times$ . (C) TPA was expressed at different time points of differentiation but not in control cells.

### 3.5 Expression and localization of *Cul4B* during differentiation

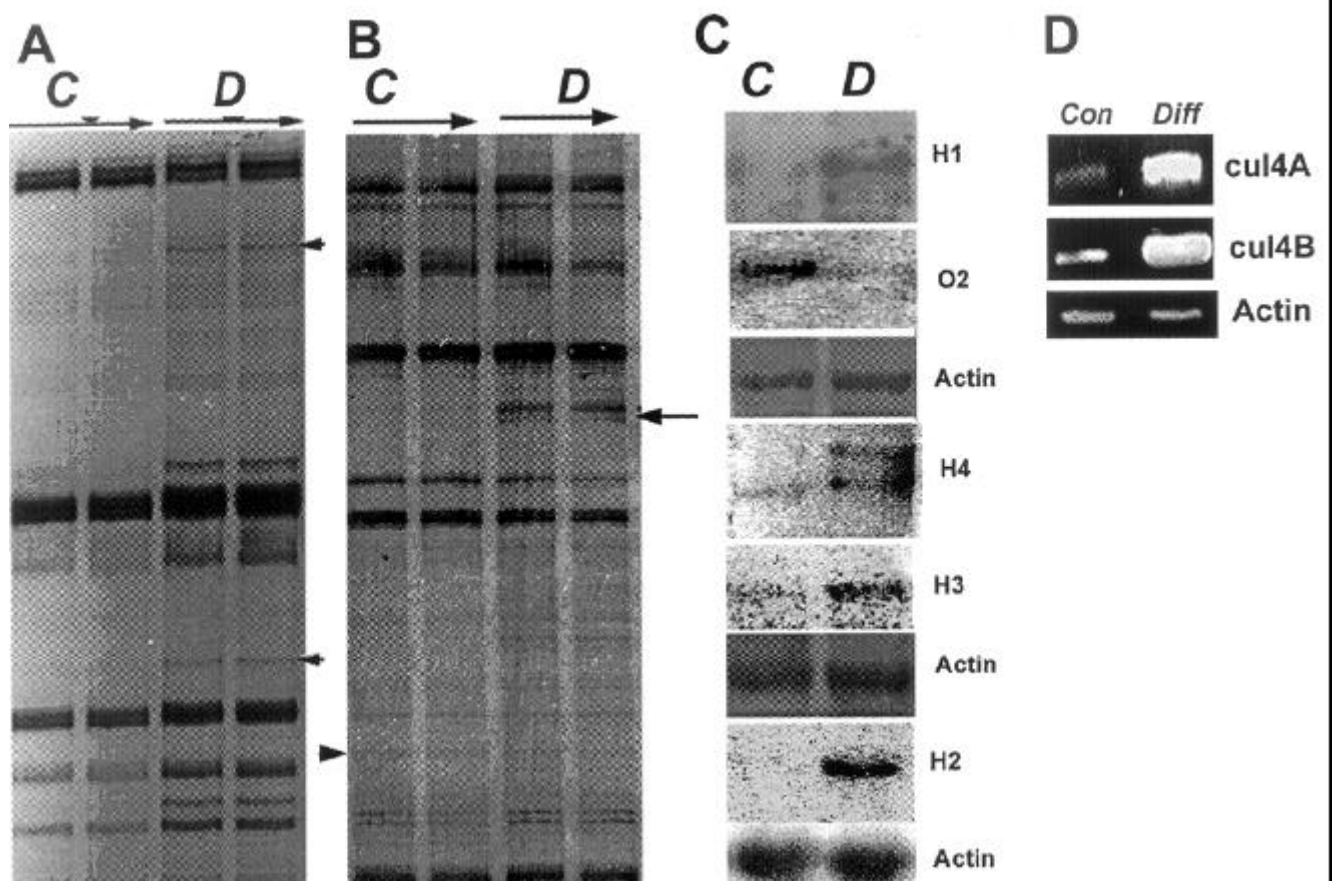
Immunoblotting with CBN antibodies further confirmed increase in *Cul4B* protein levels during differentiation of PCC4 cells (figure 5B). To confirm its enhanced expression and localization, immunofluorescence analysis of control and differentiating PCC4 cells was done with CBN and CBC antibodies. Immunofluorescence with CBN antibody showed very little *Cul4B* in the cytosol of control cells (figure 5C) whereas the amount of *Cul4B* increased immensely and was translocated into nuclei in differentiating PCC4 cells (figure 5D). Staining with CBC antibody did not show any dramatic increase in fluorescence or its translocation to the nucleus (figure 5E,F). Difference between the staining patterns of CBC and CBN can be attributed to the fact that two antibodies are raised against different epitopes. Probably antibodies against N terminus can recognize the *Cul4B* better in its native form compared to CBC antibody.

### 3.6 Functional significance of the predicted nuclear localization signal sequence

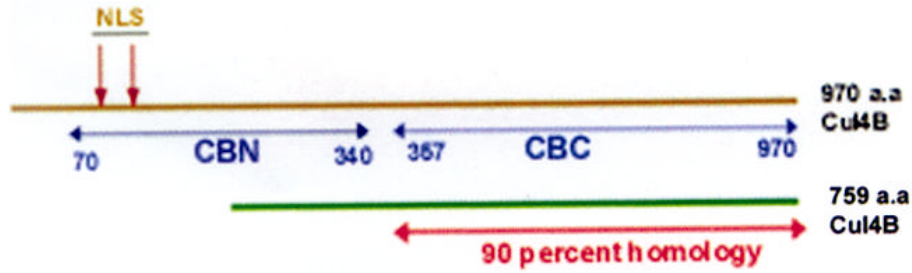
Psort program provided at ExPasy predicted two nuclear localization signal sequences (NLS) in the N-terminal region of *Cul4B* at residues 107 to 115 and 168 to 174 aa. We verified the functional significance of this prediction by fusing this part of the protein with GFP. The N-terminus fusion protein was localized exclusively to the nucleus

**Table 1.** Differentially expressed genes during PCC4 cell differentiation.

Partial c-DNA	Length	Homology
H1 (up regulated)	269	Kinesin like protein
H2 (up regulated)	312	Cullin 4A
H3 (up regulated)	250	Acc. No. 635343
H4 (up regulated)	260	Nucleosome assembly protein
O2 (down regulated)	244	Matches with an EST present in neonatal kidney (BB504873)



**Figure 3.** Identification of differentially expressed genes. (A–B) Differential display analysis of RNA from control and differentiated cells. (C) Control (duplicate loading). (D) Differentiated cells. (C) Validation of differential expression of genes expression by Northern hybridization. There were 6 true positives out of 15 such validations, 5 of which are shown here. (D) Validation of differential expression of *Cul4A* and *4B* by RT-PCR, during heat induced differentiation.



**Figure 4.** Schematic representation of Cul4B and Cul4A. NLS, nuclear localization signal sequence.

(figure 6A). In contrast, the C-terminus fused with GFP was localized only to the cytoplasm (figure 6B).

#### 4. Discussion

Early embryonic development is a complex process marked by differential expression of several genes, characteristic to each cell type in the developing embryo. Using the PCC4 cells as a model system, we have established that heat shock as well as curcumin can induce differentiation (Bisht *et al* 1994; Batth *et al* 2001). We have demonstrated expression of the endodermal marker in differentiating cells and identified several partial cDNAs that were either up or down regulated during differentiation. One of the upregulated cDNA corresponding to Cul4B was characterized.

During differentiation cell division and gene expression are strictly regulated. Several mRNAs and proteins, which are not required during differentiation, are either degraded or their amounts are finely regulated. It is possible that Cul4B, which is part of the E3 ubiquitin ligase complex, is involved in the regulation of specific proteins during differentiation.

Cullins are a family of proteins that are essential for selective degradation of different proteins involved in cell cycle regulation and signalling (Latres *et al* 1999). In mouse two closely related proteins Cul4A and Cul4B represent Cul4, coded by two different genes.

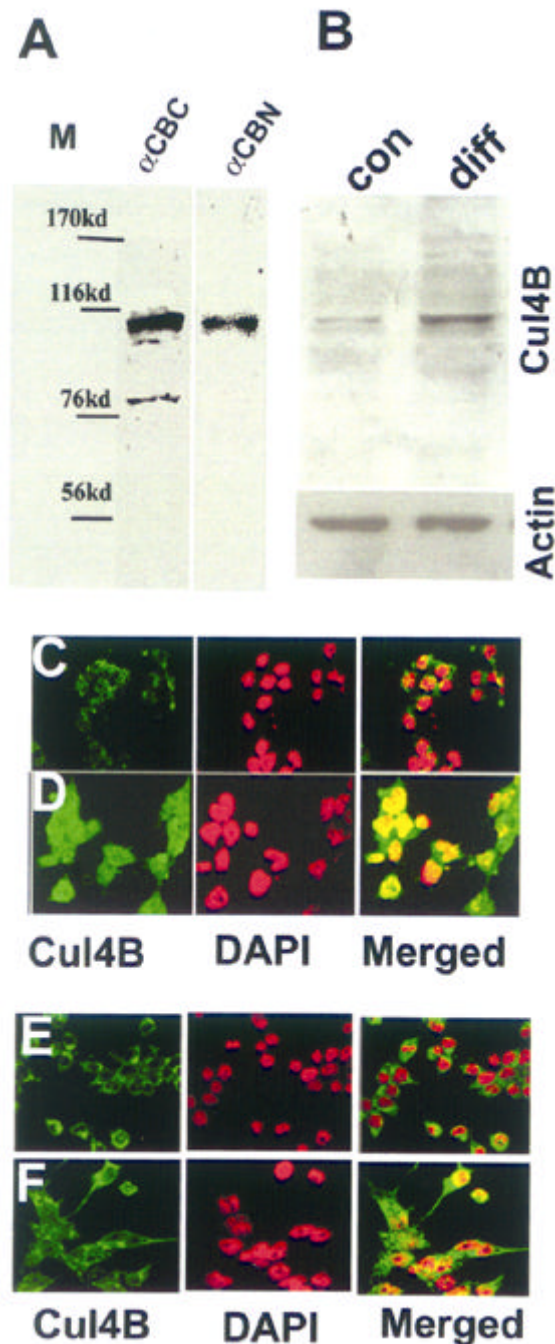
Amino acid sequence comparison of Cul4A and Cul4B revealed that Cul4B has an extended and unique N-terminus though the rest of the sequence shows 80% similarity. Despite the high similarity between these two proteins, Cul4A knockout mice are blastocyst lethal. Li *et al* (2002) demonstrated that mouse Cul4A<sup>-/-</sup> embryos implant but do not survive beyond day 7 of development even in the presence of intact Cul4B suggesting one or more distinct functions for these two proteins.

Only 2–3% of Cul4A has been reported to be present in the nucleus during UV treatment, otherwise it is always predominantly present in the cytoplasm (Chen *et al* 2001). In contrast, anti-CBN antibodies show presence of Cul4B in the nucleus as well as in the cytoplasm of PCC4 cells during differentiation. Control PCC4 cells show very little amount of Cul4B, which is present only in the cytoplasm and during differentiation it is over-expressed as well as translocated to the nucleus, suggesting a specific role for Cul4B in recognizing nuclear proteins for degradation.

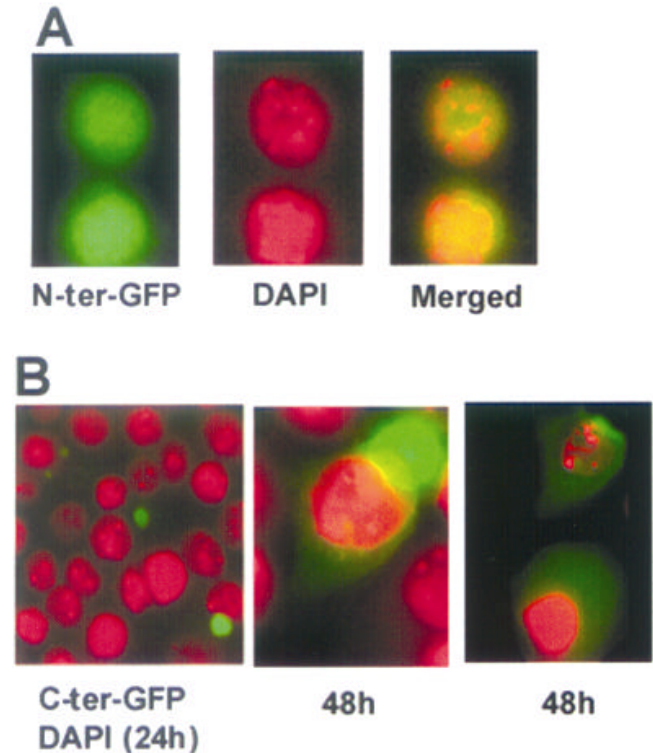
During proteasomal degradation C-terminus of Cul4 protein is probably needed for the interaction with ubiquitin conjugating enzyme (E-2) for ubiquitination of substrate proteins whereas the N-terminus is involved in interaction with F-box proteins which recognize the substrate. Presence of functional NLS in the N-terminus of Cul4B also suggests a more specific role for Cul4B as against Cul4A. Combinatorial interactions between different F-box proteins and cullins may generate a large number of E3 complexes, each with unique substrate specificity (Kitagawa *et al* 1999) for degradation of specific proteins.

To further understand the relative essentiality of Cul4B during differentiation RNAi that specifically inhibits Cul4B was designed and cloned into U6 promoter vector. Transfection of Cul4B RNAi into PCC4 cells resulted in lethality of transfected cells rather than showing differentiation specific effects (data not shown).

Deletion of different cullins has been reported to affect the development of different organisms. Cul1 is necessary for proper cell-cycle exit. In mice, loss of Cul1 results in early embryonic lethality (Dealy *et al* 1999; Wang *et al* 1999). Cul3 is required for proper development in mice since inactivation of Cul3 locus by homologous recombination results in embryonic lethality prior to 7.5 days of gestation with defects in both embryonic and extra embryonic compartments (Singer *et al*



**Figure 5.** Cul4B is up regulated during differentiation. (A) CBC antibody recognized Cul4A as well as Cul4B, whereas CBN antibody recognizes only Cul4B. (B) Western blot with total cell lysate of control and differentiated PCC4 cells shows that Cul4B is up regulated during PCC4 cell differentiation. Actin is used as loading control. (C) Control PCC4 cells were stained with CBN antibody, which showed very little expression of Cul4B in control PCC4 cells. (D) In differentiating PCC4 cells Cul4B is over-expressed and present in the nucleus as well as in the cytosol. Staining of control and differentiated PCC4 cells with CBC antibody gives signal in the (E) cytoplasm of control cells (F) in differentiated cells there is minimal increase.



**Figure 6.** Functional significance of the amino terminal NLS in Cul4B. Amino and carboxy terminal regions of Cul4B were cloned into GFP vector, transfected separately into PCC4 cells and green fluorescence of GFP was monitored in transformants. (A) N-terminal GFP fusion; notice localization of fluorescence in the nucleus (B) C-terminal GFP fusion; GFP fluorescence localized at specific regions in the cytoplasm.

1999). As discussed earlier Cul4A is also essential for embryonic development. Lethality of Cul4B RNAi transfected PCC4 cells also indicates its importance for cell survival also like other cullins.

#### Acknowledgements

The authors would like to thank Dr L S Shashidhara for the helpful discussions and critical evaluation of the manuscript, Mrs Nandini Rangaraj for help in confocal microscopy, Mr Nagesh for help in DNA sequencing and Mrs Mehar Sultana for primer synthesis. KSS was an Indian Council of Medical Research (ICMR), New Delhi sponsored PDF. The work is partly supported by ICMR Grant No. 5/13/55/99-NCD III.

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*MS received 3 November 2004; accepted 18 April 2005*

ePublication: 16 May 2005

Corresponding editor: VIDYANAND NANJUNDIAH