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Proliferation Defects and Genome Instability in Cells Lacking Cul4A

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Abstract

The Cul4A gene, which encodes a core component of a cullin-based E3 ubiquitin ligase complex, is over-expressed in breast and hepatocellular cancers. In breast cancers, over-expression of Cul4A strongly correlates with poor prognosis. Also, Cul4A is required for early embryonic development. Early lethality of mouse embryos prevented a detailed analysis of the functions of Cul4A. Here, we used a strain of mice carrying floxed alleles of Cul4A to study its role in cell division, *in vitro* and *in vivo*. Embryonic fibroblasts exhibit a severe deficiency in cell proliferation following deletion of Cul4A. We observed that the Cul4A protein is abundantly expressed in brain, liver and in the mammary tissue of pregnant mice. Deletion of Cul4A in liver impairs hepatocyte proliferation during regeneration following carbon tetrachloride induced injury. The Cul4A-deleted cells are slow in entering S phase, and are deficient in progressing through early M phase. Several cell cycle regulators, including p53 and p27Kip1, are de-regulated in the Cul4A-deleted cells. Expression of a dominant negative mutant of p53 causes significant reversal of the proliferation defects in Cul4A-deleted cells. The Cul4A-deleted cells exhibit aberrant number of centrosome, multipolar spindles and micronuclei formation. Furthermore, those cells are sensitive to UV irradiation and exhibit reduced levels of unscheduled DNA synthesis. Together, our observations indicate that Cul4A is required for efficient cell proliferation, control of the centrosome amplification and genome stability.

INTRODUCTION

Cul4 and its functional partners DDB1 (Damaged-DNA Binding protein subunit 1) and Cop9/signalosome are conserved from fission yeast to human (Bondar et al., 2006; Holmberg et al., 2005; Kim and Kipreos, 2007 and references therein). In lower organisms, the Cul4 protein is encoded by one gene, whereas in mammals two highly homologous genes encode Cul4A and Cul4B. Several conserved functions of Cul4 have been described. One that has been studied in great details is that of the proteolysis of the replication

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licensing protein Cdt1 (Reviewed in Kim and Kipreos, 2007). In mammalian cells, both Cul4A and Cul4B are able to target Cdt1 for proteolysis (Higa et al., 2003). In addition to Cdt1 proteolysis, the fission yeast homologues of Cul4 and DDB1, Pcu4 and Ddb1, are involved also in the proteolysis of the replication inhibitor Spd1, an inhibitor of the ribonucleotide reductase, which is degraded in S phase and in response to DNA damage (Holmberg et al., 2005; Bondar et al., 2004). In Drosophila cells, Cul4 is involved in the proteolysis of the cdk-inhibitor Dacapo (Higa et al., 2006). Interestingly, that function is conserved in the mammalian Cul4A protein. It has been shown that Cul4A targets p27Kip1 and p53 for proteolysis (Bondar et al., 2006; Higa et al., 2006; Li et al., 2006; Banks et al., 2006; Nag et al., 2004). In addition to p27Kip1 and p53, Cul4A has been implicated also in the proteolysis of HOXA9, the c-Jun proteins and several viral proteins (Wertz et al., 2004; Zhang et al., 2003; Ulane and Horvath, 2002; Belzile et al., 2007; Bergametti et al., 2002). However, many of the targets of mammalian Cul4A and Cul4B were identified by *in vitro* cell culture experiments. Therefore, the physiological relevance remains unclear.

The observations on p53 and p27Kip1 are significant because the Cul4A gene is amplified and overexpressed in breast and hepatocellular carcinomas (Abba et al., 2007; Chen et al., 1998; Yasui et al., 2002). Also, a recent study indicated a strong correlation between Cul4A over-expression and poor prognosis in breast cancer patients (Schindl et al., 2007). Clearly, accelerated degradation of p27Kip1 and p53 by over-expressed Cul4A is expected to support a more aggressive growth of the tumor cells. A recent study provided evidence for a role of Cul4A in the proteolysis of p27Kip1, *in vivo* (Waning et al., 2008). However, there is no clear *in vivo* evidence for a role of Cul4A in the proteolysis of p53.

Unlike the other targets of Cul4A and Cul4B, the proteolysis of p53 and p27Kip1 appears to be specific to Cul4A. Three separate studies indicated that Cul4A participates in the proteolysis of p27Kip1 (Bondar et al., 2006; Higa et al., 2006; Li et al., 2006). One of those studies indicated that the p27Kip1 proteolysis is a specific function of Cul4A, as depletion of Cul4B was unable to induce accumulation of p27Kip1 (Higa et al., 2006). The same group also reported that Cul4A, but not Cul4B, associates with Mdm2 to accelerate proteolysis of p53. Immunopurified Cul4A, but not Cul4B, increased polyubiquitination of p53, involving Mdm2 (Banks et al., 2006). The polyubiquitination of p53 by the Cul4A involves DDB1, as well as Cdt2 and PCNA (Banks et al., 2006). Thus, it appears that a Cul4A-containing complex similar to the one involved in Cdt1 proteolysis is involved in collaborating with Mdm2 to accelerate proteolysis of p53.

It was shown that Cul4A−/− embryos die soon after implantation, and no live embryo could be detected beyond 7.5 dpc (Li et al., 2002). The early lethality of the Cul4A −/− embryos precluded detailed analyses of the role of Cul4A in cell proliferation. Because of its over-expression in breast and hepatocellular carcinomas, as well as its involvement in the proteolysis of p53, we sought to investigate the roles of Cul4A in cell proliferation. Here, we have used a conditional Cul4A fl/fl strain of mice to show that Cul4A plays important roles in cell proliferation both *in vitro* and *in vivo*. Deletion of Cul4A in mouse liver causes accumulation of p53 and p27Kip1. Moreover, Cul4A-deletion retards progression through S- and early M phases of the cell cycle. Those defects were reversed by expression of a dominant negative mutant of p53. The Cul4A-deleted cells also exhibit aberrant number of

centrosome, multipolar spindles, micronuclei, increased sensitivity to UV and deficient nucleotide excision repair. These observations provide evidence for roles of Cul4A in both cell proliferation and genome stabilization.

RESULTS

Proliferation defects in the Cul4A-deleted cells

Because Cul4A is over-expressed in tumors, we sought to investigate its role in cell proliferation. We generated a strain of mice harboring floxed alleles of Cul4A, as described in the Materials and Methods. A genomic PCR (Fig. 1A upper panel) is used to identify the mice with Cul4A fl/fl alleles. To confirm conditional deletion of the gene in the presence of Cre recombinase, we generated embryonic fibroblasts (MEFs) and infected them with recombinant adenovirus expressing Cre recombinase. Forty-eight and seventy-two hours after infection, cells were harvested, and the extracts were analyzed for the levels of Cul4A using a Cul4A-specific antibody. Expression of Cre recombinase resulted in a near complete loss of the Cul4A protein (Fig. 1A lower panel), as expected from the Cre-mediated deletion of the floxed Cul4A sequences. To investigate the effects of the loss of Cul4A on proliferation, the infected cells were then re-plated at equal density. The cells were plated in 6-well plates at 10^5 cells per well, which is the optimal density for MEFs under our culturing conditions. The cell number was counted each day for 6 days. Representative data are shown in Fig. 1B. There was no significant difference in the levels of the dead cells. But, the Cul4A-deleted MEFs exhibited a significantly slower rate of proliferation compared to the undeleted MEFs (infected with Ad-LacZ), suggesting that the loss of Cul4A impairs cell proliferation. The difference in proliferation-rate did not result from Cre expression alone because the wild type MEFs or Cul4A+/fl MEFs exhibited no difference in proliferation when infected with Ad-Cre and Ad-LacZ (Supplemental Fig. 1). Also, we did not detect any increase in premature senescence of the Cul4A-deleted MEFs after 6 days in the culture.

Analysis for distribution of cells in different phases by flow cytometry did not show any significant difference between the Ad-Cre and Ad-LacZ infected cells (Supplemental Fig. 2), suggesting that deletion of Cul4A affects progression through multiple phases, if not all phases, of the cell cycle. Therefore, we investigated progression of the Cul4A-deleted cells through the different phases. To analyze the entry into S phase, MEFs were infected with Ad-Cre or Ad-LacZ and maintained in serum-free medium for 60h. A similar level of G0/G1 arrest was observed for both samples (data not shown). Following serum starvation, the medium was replenished with 20% fetal bovine serum, and at different time-points cells were pulsed with 3μg/ml of BrdU for 1h. The cells were fixed and subjected to immunostaining to measure the rate of BrdU-incorporation. As shown in Fig. 1C, the LacZ expressing cells entered S phase at around 13h following serum stimulation and the BrdU incorporation rate peaked at around 16h following the stimulation. The Cul4A-deleted cells, on the other hand, exhibited a clear delay in entry into S phase. Moreover, rate of incorporation was impaired, peaked at around 20h following serum stimulation, indicating both entry and progression through S phase are slower in cells lacking Cul4A. To investigate the progression through the G2-M phases, the Cre or LacZ expressing MEFs were synchronized at the G1/S boundary by double-thymidine block. Following release, the entry

into M phase was measured by assaying for phosphorylated-histone H3 (H3P) positive nuclei by immunostaining. The Cul4A-deleted cells exhibited a delay in the M phase, as judged by H3P positive cells (Fig. 1D). To investigate a deficiency in M phase progression, the cells were arrested in early M phase by treatment with nocodazole, as described in the Materials and Methods. The M phase cells were harvested by mitotic shake-off, and released from M phase-arrest by plating them in medium without nocodazole. Forty-five minutes after release, the cells were fixed and subjected to immunostaining with antibody against alpha-tubulin, and co stained with DAPI. The cells at different phases were quantified by analyzing at least 100 mitotic cells. As shown in Fig. 1E, there was a significant increase in number of cells in prometaphase in Cul4A deficient cells, suggesting that Cul4A is required for efficient progression through early mitosis. Together, our observations suggest that Cul4A is required for proper progression through multiple phases of the cell cycle.

Inhibition of p53 reverses proliferation defects in the Cul4A-deleted cells

In human tumor cell lines, Cul4A has been shown to participate in the proteolysis of p53 and p27Kip1 (Bondar et al., 2006; Higa et al., 2006; Li et al., 2006; Banks et al., 2006; Nag et al., 2004), which are potent inhibitors of the cell cycle. Consistent with those observations, extracts of the Cul4A-deleted MEFs exhibited higher levels of p53 and p27Kip1 compared to the extracts from the control MEFs (Fig. 2A). As expected, they also had higher level of p21Cip1. We did not detect any difference in the levels of DDB1 (not shown). The accumulation of p53 resulted from a deficiency in the proteolysis because the decay rate of p53 was slower in Cul4A-deleted cells (Fig 2B). The decay rates of p53 were measured by adding cycloheximide to the culture medium to inhibit new protein synthesis. In the LacZ expressing cells, p53 exhibited a high decay rate and the half-life was less than 10 min, whereas in the Ad-Cre infected cells, the half-life of p53 was about 20 min. Also, there was a significant increase in the half-life of p27Kip1 in cells lacking Cul4A (Fig. 2B). However, we failed to detect any significant stabilization of p21Cip1 (Fig. 2B). To determine whether the accumulation of p21Cip1 resulted from an increased expression, we assayed for and compared the mRNA levels of p21Cip1 in the serum stimulated MEFs. The p21Cip1-mRNA was assayed by quantitative RT-PCR assays. Clearly, the Cul4A-deleted cells, compared to the LacZ expressing cells, expressed p21Cip1-mRNA at a much higher level (Fig. 2C). We speculate that the increase in p21Cip1-mRNA expression could be a result of higher levels of p53 in the Cul4A-deleted cells.

To investigate the role of the p53-p21Cip1 pathway on proliferation defects observed in Cul4A-deleted cells, we expressed a dominant negative mutant of p53 in the Cul4A fl/fl MEFs. The MEFs were then infected with Ad-Cre to delete Cul4A or with Ad-LacZ. The infected cells were subjected to proliferation assays. There was a significant reversal of rate of cell growth. The Cul4A-deleted and undeleted cells exhibited a very similar growth-curve (Fig. 3A). To compare S phase entry, the cells were synchronized by serum-starvation and then supplemented with medium containing serum, and BrdU-incorporation rate in 1h pulse was assayed by immunostaining. The BrdU-incorporation peaked at about the same time, and the rates of incorporation were comparable (Fig. 3B). Moreover, the delay in progression through early M phase was abolished by the expression of dominant negative

p53 (Fig. 3C). Together, these observations suggest that the proliferation defects in Cul4A-deletedcells is largely linked to p53 accumulation.

Cul4A deficiency leads to defective proliferation of mouse hepatocytes following CCl_4 liver injury

We observed that the mouse Cul4A protein is abundantly expressed in the adult liver (Fig. 4A). Therefore, we wanted to investigate the role of Cul4A in cell proliferation in adult mouse liver during regeneration. The Cul4A fl/fl mice were crossed with a transgenic line (Alb-Cre) that expresses Cre specifically in the liver. The Alb-Cre strain was shown to bring about a deletion of floxed alleles by 6 weeks following birth (Postic and Magnuson, 2000). We compared livers isolated from male mice, 10−11 weeks of age, expressing Cre (Cul4A fl/fl Alb-Cre) with those without Cre expression (Cul4A fl/fl). The extracts from three different pairs of livers were prepared (pre-cleared for IgG by 4hrs incubation with 50% slurry of Protein A-Agarose and Protein G-Sepharose) and analyzed for the levels of p53 and p27Kip1. As can be seen in Fig. 4B, expression of Cre caused a reduction in the levels of Cul4A. The loss of Cul4A coincided with an increase in the levels of p27Kip1 and p53, thus providing *in vivo* support for the notion that Cul4A is involved in the proteolysis of p53 and p27Kip1. We observed five male mice with Cul4A deletion in the liver for about eleven months. One of them died at 6 months of age, while three out of the remaining four exhibited 23.38±4.2% weight reduction as time progressed compared to control littermates (data not shown).

Liver is an excellent model for studying proliferation *in vivo*. Following tissue loss (physical or chemical injury), it has ability to regenerate and restore to appropriate size. Carbon tetrachloride (CCl_4) has been widely used to induce chemical injury of the liver. It induces necrosis of pericentral hepatocytes (Fausto, 1996). In response to tissue loss of pericentral region, the hepatocytes within periportal and intermediate zones proliferate (Serfas et al., 1997; Fausto, 1996). To investigate hepatocyte proliferation in the Cul4A deficient mouse liver, we subjected the Cul4A f/fl Alb-Cre mice to carbon tetrachloride liver injury following a previously described procedure (Serfas et al., 1997). Cul4A fl/fl strain was used as the control group. Briefly, the mice were injected with carbon tetrachloride in corn oil or corn oil alone. At different time-points following carbon tetrachloride injection, the mice were sacrificed. Two hours prior to sacrifice, the mice were subjected to i.p. injection with BrdU (50 μg/g of animal weight) to score for DNA replication. The livers were harvested, and sections were prepared, as described in the Materials and Methods. The sections were subjected to immunohistochemical staining for BrdU-positive nuclei using a monoclonal antibody against BrdU. For a count of the total number of nuclei, sections were also stained with Vector's Nuclear Fast Red. The BrdU-positive nuclei from three different mouse livers were quantified in total of 1000 nuclei from at least five different fields. As can be seen in Fig. 4C, the control group exhibited abundant BrdU-positive cells between 36 and 48hrs following carbon tetrachloride treatment. The Cul4A-deficient liver sections exhibited a clear deficiency in BrdU-incorporation, suggesting a deficiency in S phase entry, as well as lower rate of progression through S phase. A representative data corresponding to the 36h time-point is shown in Fig. 4D. A significant difference also in the number of mitotic figures in the H & E stained sections were observed (Fig. 4D, lower panel). We measured the

necrotic lesions (both number and size) in the H & E stained liver sections. Overall, the number of lesions was comparable between the Cul4A-deleted and undeleted samples, and at the 24h time-point, the sizes of the lesions were also very comparable (Supplemental table 1). At the later time-points, larger lesions were more frequent in the liver sections of the Cul4A-deleted mice, which would be consistent with a delay in regeneration in the absence of Cul4A.

Evidence for chromosomal instability in Cul4A-deleted MEFs

DDB1 partners with both Cul4A and Cul4B to form distinct E3 ligase complexes. A recent study indicated that DDB1 is required for genome stabilization (Cang et al., 2006). It was shown that the MEFs lacking DDB1 exhibited centrosome amplification and micronuclei at high frequency. We investigated whether Cul4A participates in those cellular functions of DDB1 that are related to genome stability. To study centrosomal aberrations, we infected Cul4A fl/fl MEFs with adenovirus expressing LacZ or Cre. Twenty-four hours after infection, cells were plated at equal density and maintained in 10% FBS containing medium. After a total of 60−72 h after infection, cells were fixed and stained with specific antibody against centrosomal marker γ-tubulin and DAPI (Fig. 5A). We examined 300 cells and counted the ones with three or more signals for γ-tubulin. The cells lacking Cul4A exhibited increased number of γ-tubulin signals at much greater frequency compared to the control (Fig. 5A, lower panel). As shown in Fig 5B, loss of Cul4A also increases incidence of multipolar spindles. To enrich mitotic population of cells and examine the spindles, cells were synchronized by double thymidine block after infection, released 8−10h after and stained with γ-tubulin antibody. Representative data from MEFs isolated from 3 different embryos is shown (Fig 5B). At least 50 mitotic spindles from each embryo were analyzed.

Formation of multipolar spindles leads to unequal segregation of chromosomes during mitosis and often in appearance of micronuclei. To investigate possible role of Cul4A in micronuclei formation, we examined the cells stained with DAPI (Fig. 5C). Examinations of the DAPI-stained cells provided clear evidence for micronuclei in the Cul4A-deleted cells. We examined at least 300 cells from each infection for micronuclei. A quantification of the results is shown in Fig. 5C, lower panel. The Cul4A-deleted MEFs consistently exhibited 2.5 fold more micronuclei over the control set. The extent of polyploidy in our FACS analyses (Supplemental Fig. 2) was less because of the insensitivity of the assay. Together, these results further confirm a role of the Cul4A-DDB1 complex in maintaining genome stability through proper segregation of the chromosomes.

Sensitivity to UV irradiation and deficiency in unscheduled DNA synthesis in Cul4A-deleted MEFs

Because of genomic instability, we sought to investigate whether the Cul4A-deleted cells are more sensitive to UV or ionizing radiation. We infected the Cul4A fl/fl MEFs with Ad-Cre or Ad-LacZ. Twenty-four hours following infection, the cells were re-plated at equal density. After another 24hrs, the cells were subjected to UV irradiation (4 or 8 J/m^2) or ionizing radiation (5 or 10 Gy). The treated cells were maintained for 48hrs following the treatment. The dead cells were identified by the trypan blue exclusion method. We did not observe significant difference in cell death between the Ad-Cre and Ad-LacZ infected cells

when treated with ionizing radiation (data not shown). On the other hand, UV irradiation reduced viability of the Ad-Cre infected cells more effectively than the Ad-LacZ infected cells (Fig. 6A). The sensitivity to UV irradiation could be related to higher levels of p53 in the Cul4A-deleted MEFs because those cells expressed the p53 target genes at higher levels (Fig. 6B). Higher levels of Bax expression could explain the sensitivity of the cells to UV irradiation. It is noteworthy that the Cul4A-deleted cells exhibited higher level of both total p53 and p53 phosphorylated at Ser18 residue (Fig. 6B).

Cul4A associates with the nucleotide excision repair (NER) protein DDB2, encoded by the XPE gene (Nag et al., 2001). Recent studies suggested that a complex of Cul4A-DDB1 and DDB2 are important for NER, as siRNA mediated knockdown of Cul4A in human cancer cell lines reduces the extent of NER (El-Mahdy et al., 2006). We investigated the role of Cul4A in NER by assaying unscheduled DNA synthesis (UDS), which measures H3-thymidine incorporation in the DNA of non-S phase cells, as a function of UV irradiation (Smith et al., 2000). The Cul4A fl/fl MEFs were infected with Ad-Cre or with Ad-LacZ. The infected MEFs were treated with H3-thymidine for 1hr to identify and distinguish the S phase cells (the S phase nuclei undergo dense labeling, Smith et al., 2000). The cells were then subjected to UV irradiation (12 J/m^2), and maintained in medium containing H3-thyimidine for 3hrs in the absence of serum. The medium was then changed to medium containing unlabeled thymidine. After 1hr, the cells were fixed, treated with the photographic emulsion EM-1 (Amersham) and developed for the measurement of UDS. The UDS was measured by counting grains per nucleus of the non-S phase cells. As expected, majority of the Cul4A-deleted cells exhibited fewer grains per nucleus compared to the control set (Fig. 6C, upper panel). A quantification of the number of grains for 30 nuclei is plotted (Fig. 6C, lower panel). These results further confirm a role of Cul4A in NER.

DISCUSSION

The Cul4A gene in chromosome 13q34 is amplified in cancers (Chen et al., 1998; Yasui et al., 2002). Also, there is a strong correlation between over-expression of Cul4A and short survival of breast cancer (Schindl et al., 2007). In this study, we provide evidence that Cul4A is critical in cell proliferation. The Cul4A-deleted MEFs are deficient in progression through S phase and early M phase. The deficiencies in the cell cycle progression could be explained by the accumulation of p53 and the cell cycle inhibitors p21Cip1 and p27Kip1. Inhibition of p53 alone caused a significant reversal of the proliferation defects in Cul4A-deleted cells, suggesting that Cul4A promotes cell proliferation partly by accelerating the proteolysis of p53. These observations provide mechanistic insights into the significance of Cul4A over-expression in breast and liver cancers.

We found that Cul4A is expressed at higher levels in brain, liver and in the mammary tissue of pregnant mice. We studied the role of proliferation using a liver specific knockout system. It is noteworthy that an amplicon containing the Cul4A gene is amplified also in human hepatocellular carcinoma (Yasui et al., 2002). Consistent with the cell culture studies, deletion of Cul4A in the liver leads to the accumulation of p53 and p27Kip1, providing *in vivo* evidence for Cul4A-dependent proteolysis of p53 and p27Kip1 in the

mouse liver. Moreover, our results provided in vivo evidence for a requirement for Cul4A in hepatocyte proliferation.

It was shown that MEFs lacking DDB1, the adapter for Cul4A, exhibit aberrant centrosome amplification (Cang et al. 2006). We found that the Cul4A-deleted MEFs also exhibit aberrant number of centrosomes, as judged by increase in number of γ-tubulin signals. The centrosome number in cell is controlled by a variety of regulators, including the tumor suppressors p53 and BRCA1 (Fukasawa, 2005; Shinmura et al., 2007). Moreover, at G1/S phase boundary, cyclinE/cdk2 plays important role in regulated duplication of the centrosome (Fukasawa, 2005). Centrosomes are critical for equal segregation of the chromosomes during mitosis. The presence of more than two centrosomes can promote formation of multipolar spindles. Indeed, Cul4A deleted MEFs exhibited high incidence of multipolar spindles. It is well documented that aberrant spindles can lead to an imbalance of chromosome segregation and eventual aneuploidy, a hallmark of genome instability and cancer. The imbalance in chromosome segregation often leads to the formation of micronuclei, parts of chromosomes that are not incorporated into the nucleus during mitosis. We observed that the Cul4A-deleted MEFs contained micronuclei at a high frequency. These observations in Cul4A-deleted cells are similar to what was observed with DDB1-deleted MEFs (Cang et al., 2006). Therefore, it is likely that the Cul4A-DDB1 complex participates in regulating the number of centrosomes. Because of accumulation of p53 and the cdk-inhibitors, which are regulators of centrosome amplification, in the Cul4A-deleted cells, we think that the mechanism by which Cul4A-DDB1 regulates the centrosome number is independent of p53 and the cdk-inhibitors. Also, inhibition of p53 by expression of a dominant negative mutant of p53 did not restore the centrosome-defects in the Cul4A-deleted cells (data not shown).

The sensitivity of the Cul4A-deleted cells to UV irradiation is expected from the high levels of p53 in those cells because p53 induces expression of several pro-apoptotic proteins, including Bax. Deficiency in nucleotide excision repair (NER) of the UV-damaged DNA also reduces viability of fibroblasts. Cul4A-associated proteins DDB1 and DDB2 have been implicated in NER (El-Mahdy et al., 2006). It is interesting that Cul4B in the Cul4A-deleted cells fails to compensate for the loss of Cul4A in NER, suggesting that a Cul4A-specific pathway is involved in that process.

MATERIALS AND METHODS

Generation of Cul4A fl/fl mice

Mice harboring floxed alleles of Cul4A were generated in collaboration with Xenogen Biosciences. Briefly, a vector containing floxed Cul4A exons (exons 4 to 8) along with a floxed NeoR gene and a negative selection marker was used for gene targeting in ES cells. The ES cells harboring a floxed allele of Cul4A and deleted NeoR were confirmed by Southern blotting and genomic PCR. Blastocyst injection was performed for 2 ES clones to obtain chimeras. From breeding of chimeras with C57BL/6 Taconic wildtype mice, heterozygotes mice were obtained. The heterozygotes were bred to generate a strain with homozygous Cul4A fl/fl alleles. Genomic DNA isolated from tails was used for genotyping

with following primers : 5 ’-gtgtttaaacGACCACAGCACACAGTAAGTAAGCCCT-3 ’ and 5 ’-gtctcgagATGAAGACATGGGTGGACAGTGGC-3’ at 58°C as annealing temperature.

Cell culture and Adenovirus infection

All experiments were performed with MEFs with passage numbers 2 to 4. Cells were infected with adenoviruses (25 plaque-forming units per cell) expressing LacZ, as a control virus, or with Ad-Cre. For Cre-mediated deletion of Cul4A cells were harvested 48−72hrs after infection.

BrdU incorporation

For proliferation assay, Cul4A fl/fl MEFs were infected with AdLacZ or AdCre virus and 24hrs after, re-plated on glass coverslips. At indicated time points, asynchronously growing MEFs were pulse labeled with 3μg/ml BrdU for 4hrs and fixed with 70% ethanol. To investigate entry into the S phase, Cul4A fl/fl MEFs were infected with adenoviruses expressing LacZ or Cre and kept in serum-free medium for 60hrs. Cells were re-plated and stimulated to re-enter the cell cycle by addition of 20% FBS in DMEM. At each time point, cells were pulsed for 1hr with 3μg/ml BrdU, fixed followed by incubation overnight with mouse anti-BrdU (Dako; dilution 1:200). After rinsing with PBS, cover-slips were incubated with FITC-conjugated polyclonal rabbit anti-mouse antibody and counterstained with DAPI.

p53, p21Cip1 and p27Kip1 decay rate

48hrs after infection with AdLacZ or AdCre, Cul4A fl/fl MEFs were pooled and re-plated. 24hrs after, cells were treated with 50μg/ml cycloheximide and harvested at indicated time points. Cells were lysed in buffer containing 0.4M NaCl, 20 mM Tris-HCl, pH 7.5, 0.1% NP40, 5% (V/V) glycerol, 1 mM NaF, 1 mM Na-orthovanadate and protease inhibitor cocktail. Extracts were subjected to polyacrylamide-SDS gel electrophoresis followed by blotting to nitrocellulose. Anti-cullin4A antibody has been previously described (Bondar et al., 2006). Anti-p21 and anti-p27 were purchased from BD Pharmingen. Anti-p53 antibody was from Santa Cruz Biotechnology, as well as anti-Cdk2 and anti-tubulin, while anti-p53Ser18P was from Calbiochem.

RT-PCR analysis

One microgram of the total RNA was subjected to DNase I treatment using RQ1 RNase-free DNase I (Invitrogen). The DNase I-treated RNA was then reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. PCR amplification was performed in triplicate using the following primers: *p21* , 5'-TTCCGCACAGGAGCAAAGTG-3' and 5'-AAGTCAAAGTTCCACCGTTCTCG-3' (annealing temperature, 64°C) and GAPDH, 5'-AACTTTGGCATTGTGGAAGG-3' and 5'-CCATCCACAGTCTTCTGGGT-3' (annealing temperature, 60°C). Each PCR mix contained: 0.05 μg of cDNA, a 100 nM concentration of each primer, and 1x iQ SYBR green supermix (Bio-Rad) in a 25-μl reaction mix. Real-time PCR was performed using the MyiQ single-color real-time PCR detection system (Bio-Rad). Melting curve analysis was performed for every reaction, and a single sharp peak was observed. The levels of *p21*

mRNA were normalized against the levels of GAPDH mRNA that was used as internal control.

CCl_4 induced liver injury

Cul4A fl/fl as a control and Cul4A fl/fl AlbCre male mice 8−11 weeks of age were injected intraperitoneally with 10 μl/g of body weight of a 10% solution of CCl_4 in corn oil, or corn oil alone (0hr). Mice were sacrificed at indicated time points. 2hrs before sacrificing, mice were ip injected with 50 μg/g of animal weight of BrdU dissolved in PBS. Livers were fixed in formalin overnight and paraffin embedded for sectioning. Sections were stained with H&E and anti-BrdU (Dako 1:200), as described previously (Serfas et al., 1997).

Unscheduled DNA synthesis (UDS) assay

The UDS assay was performed following previously described procedures (Smith et al., 2000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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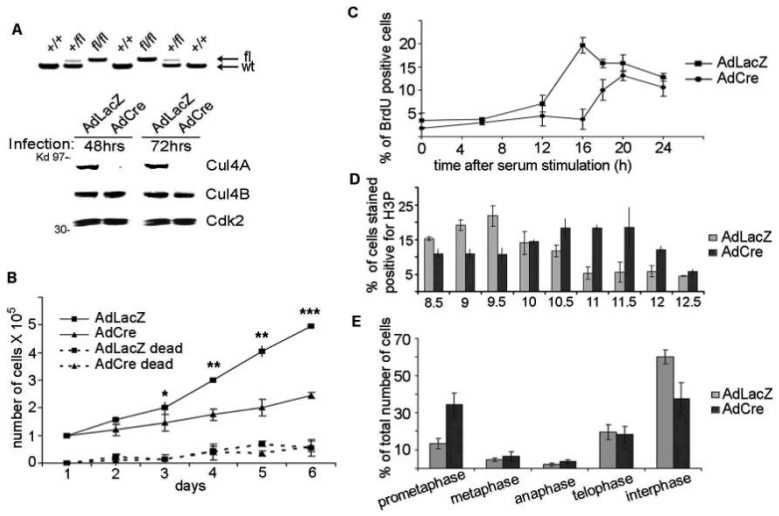

Fig.1. Proliferation defects of the Cul4A-deleted MEFs

(a) We generated a strain of mice harboring floxed alleles of Cul4A (Cul4A fl/fl). A genomic PCR assay for the floxed- and the wild type allele is shown. To confirm the Cre recombinase mediated deletion of the Cul4A gene, fibroblasts (MEFs) from the Cul4A fl/fl embryos were infected with adenovirus expressing LacZ or Cre. The infected cells, after 48h or 72h, were harvested and the extracts (0.15 mg) were assayed for the level of Cul4A or Cul4B in western blots using specific antisera. Cdk2 was assayed as a loading control. (b) To measure proliferation, the Cul4A fl/fl MEFs were infected with Ad-LacZ or Ad-Cre. Twenty-four hours after infection, the cells were re-plated at equal density (1×10^5). The cell number is counted every day for six days. An average from three experiments is plotted. (c) The Cul4A fl/fl MEFs were infected with Ad-LacZ or Ad-Cre, and the cells were maintained in serum free medium. After 60h, the cells were pooled and re-plated at equal density using medium with fetal bovine serum (20%). At the indicated time-points, BrdU (3μg/ml) was added to the culture medium for 1h. The cells were fixed and subjected to immunostaining for BrdU with a monoclonal BrdU-antibody. The cells were stained also with DAPI. The percent BrdU-positive cells from three experiments is plotted. (d) The Cul4A-deleted and undeleted cells were synchronized by double thymidine block. At the indicated time-points, following release from the block, the cells were fixed and subjected to immunostaining for H3P. A quantification of the data is shown. (e) The Cul4A fl/fl MEFs were infected with Ad-LacZ or Ad-Cre. Forty-eight hours after infection, nocodazole (100 ng/ml) was added to the culture medium. After 12h of nocodazole treatment, the M phase cells were harvested by mitotic shake off, and re-plated in medium without nocodazole. Forty-five min after replating, the cells were fixed and subjected to immunostaining with alpha-tubulin antibody. The cells were stained also with DAPI. The cells at various sub-phases in mitosis were examined by fluorescent microscopy (upper panel). A quantification of the cells at different sub-phases is shown (lower panel). The asterisk in panel B indicates statistically significant differences, with the following P values calculated by the Student *t* test: *, $P<0.05$; ** $P\leq0.01$; ***, $P\leq0.001$.

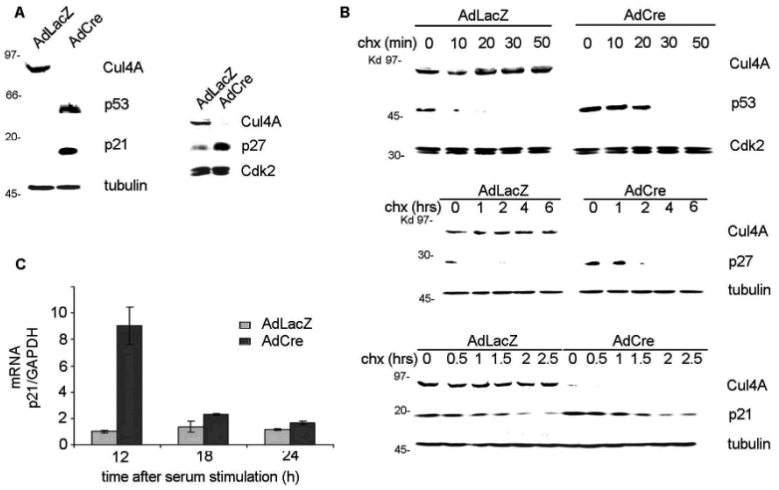

Fig. 2. Stabilization of p53 and p27Kip1 and increased expression of p21Cip1 in Cul4A-deleted cells

(a) The Cul4A fl/fl MEFs were infected with Ad-LacZ or Ad-Cre for 60h. Extracts (0.15 mg) of the infected cells were analyzed by western blots for p53, p27Kip1 and p21Cip1. (b) To measure the decay-rates of p53, p21Cip1 and p27Kip1, the infected cells were treated with cycloheximide (50 μg/ml). The cells were then harvested at the indicated time-points. Extracts (0.15 mg for p53, 0.1 mg for p27Kip1 and p21Cip1) were analyzed by western blot assays. Cdk2 or alpha-tubulin was assayed as a loading control. (c) Ad-LacZ or Ad-Cre infected cells were serum starved and then stimulated by adding serum. Total RNA from cells at the indicated time-points following serum stimulation was isolated and subjected to quantitative-RT-PCR assays for the levels of p21Cip1, as described in the Materials and Methods.

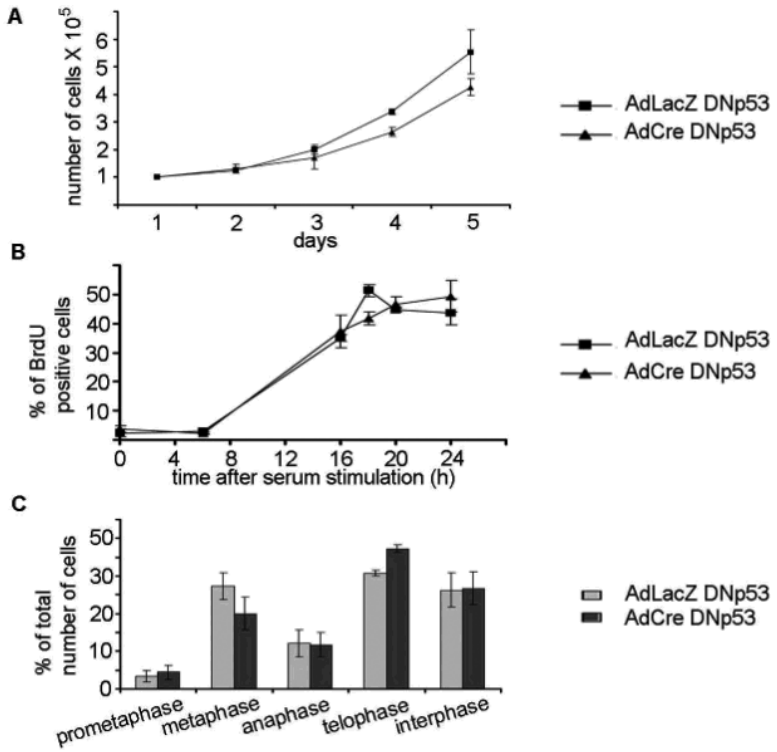

Fig. 3. Reversal of proliferation defects in Cul4A-deleted cells by dominant negative p53

Cul4A fl/fl MEFs were infected with retrovirus expressing a dominant negative mutant of p53 (dNp53). Cells expressing the dNp53 were infected with Ad-Cre or Ad-LacZ. The infected cells were subjected to growth-curve analyses (a), S-phase entry and progression after synchronization at the G0/G1 phases (b) and M phase progression after release from nocodazole arrest (c), as described in the legend to Fig.1.

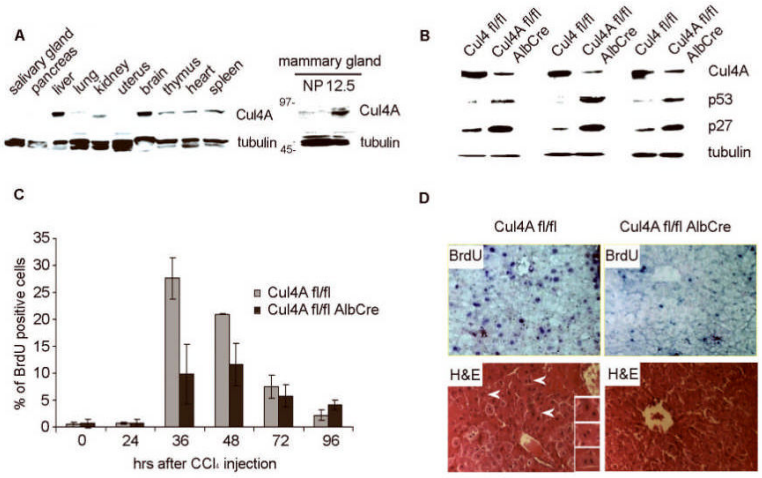

Fig.4. Deficiency in hepatocyte proliferation in Cul4A-deleted liver

(a) Left: extracts (0.2 mg) from various mouse tissues were assayed for the level of Cul4A by western blot assay. Alfa-tubulin was assayed as a loading control. Right: protein extract from virgin (NP) and 12.5 days pregnant (P) female mice were analyzed for Cul4A expression. (b) The Cul4A fl/fl mice were crossed with a transgenic strain expressing Cre recombinase driven by the albumin promoter (Alb-Cre) to generate Cu4A fl/fl Alb-Cre bi-transgenics. Male mice at 10−11 weeks of age were used in this experiment. Livers from three mice of Cul4A fl/fl background or from the bi-transgenic line were harvested and extracts were first depleted of IgG by incubating with Protein A- and protein G- Sepharose beads. The extracts (0.2 mg) were assayed for p53, p27Kip1 and tubulin by western blot assays. (c) & (d) Mice (8 to 11 weeks of age) were injected with carbon tetrachloride dissolved in corn oil as described in the Materials and Methods. At least three mice were used per time-point. Two hours before sacrificing, the mice were injected with BrdU. Livers were harvested, paraffin embedded, sectioned and subjected to immunohistochemical staining with BrdU-antibody. The sections were also stained with hematoxillin and eosin (H&E). A quantification of percent BrdU-positive cells is plotted (c). A representative immunohistochemical staining for BrdU and H&E staining of the 36h sample is shown (d). The arrows in lower panel of d indicate mitotic figures, which are shown also in the inset.

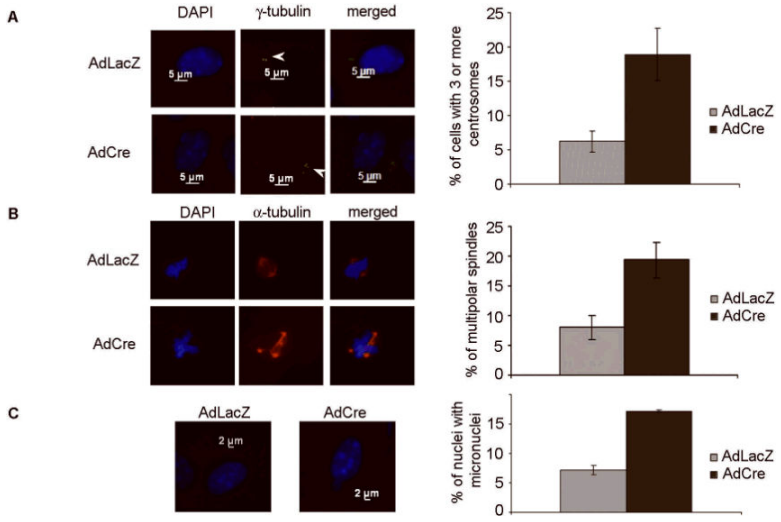

Fig. 5. Centrosomal aberration, multipolar spindle and micronuclei formation of Cul4A-deleted cells

(a) Cul4A fl/fl MEFs were infected with Ad-LacZ or Ad-Cre for 60h, fixed and subjected to immunostaining with γ-tubulin. The cells were also stained with DAPI. The experiment was performed three times in triplicates. A quantification of the percent cells containing more than three centrosomes from representative experiment is shown. (b) To analyze mitotic spindles, the cells were synchronized to G1/S boundary by double thymidine-block and then released from block. After 8−10h, cells were fixed and subjected to immunostaining with alfa-tubulin antibody, as well as to staining by DAPI. A quantification of the cells with multipolar spindles is shown. (c) About 300 DAPI stained cells were also examined for micronuclei. Quantification from representative experiment is shown.

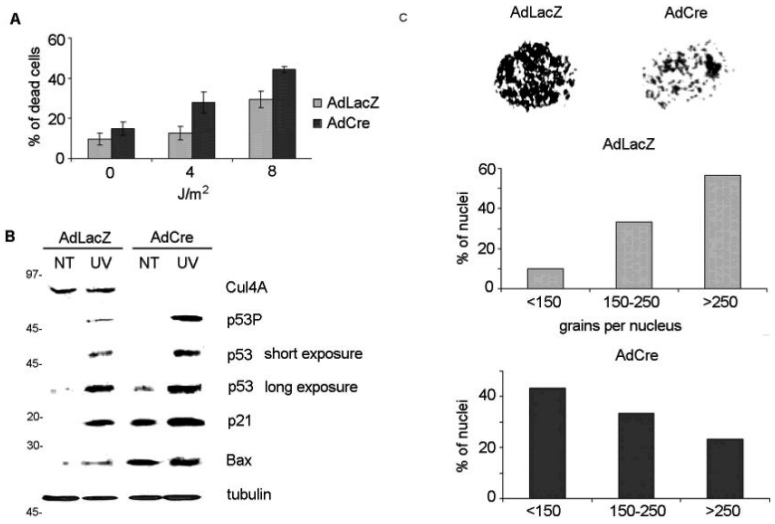

Fig. 6. Cul4A-deleted cells are sensitive to UV irradiation and deficient in unscheduled DNA synthesis (UDS)

(a) Cul4A fl/fl MEFs were infected with Ad-LacZ or Ad-Cre for 24h. The cells were pooled and re-plated at equal density. Twenty-four hours after re-plating, the cells were exposed to 4 or 8 J/m2 of UV irradiation. Forty-eight hours after irradiation, the viability was measured by the trypan blue exclusion method. A quantification of the dead cells from three experiments is shown. (b) Extracts (0.2 mg) from UV irradiated (12 J/m2) or unirradiated cells were analyzed for the levels of p53, p53Ser18P (indicated as p53-P), p21Cip1 and Bax using specific antibodies in western blot assays. (c) Unscheduled DNA synthesis (UDS) was measured in the Cul4A fl/fl MEFs infected with Ad-LacZ or Ad-Cre, following a previously described procedure (Smith et al., 2000). The slides were developed for a week. After development, the grains per nucleus were obtained by counting 30 non-S phase nuclei per sample. Representative autoradiographs of the grains in the nucleus of the Ad-LacZ and the Ad-Cre infected cells are shown in the upper panel. Grains per nucleus are plotted in the lower panel.