Loss of the anaphase-promoting complex in quiescent cells causes unscheduled hepatocyte proliferation

Karin G. Wirth, Romeo Ricci, Juan F. Giménez-Abián, et al.

Access the most recent version at doi:10.1101/gad.285404

**Supplemental Material**
http://genesdev.cshlp.org/content/suppl/2013/07/02/18.1.88.DC1.html

**References**
This article cites 47 articles, 30 of which can be accessed free at:
http://genesdev.cshlp.org/content/18/1/88.full.html#ref-list-1

**Email Alerting Service**
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.
Loss of the anaphase-promoting complex in quiescent cells causes unscheduled hepatocyte proliferation


1Research Institute of Molecular Pathology (IMP), A-1030 Vienna, Austria; 2Institute of Physiology, Cardiovascular Research, University of Zürich and Department of Cardiology, University Hospital Zürich, Zürich, Switzerland; 3Institute of Clinical Pathology, University Hospital Zürich, CH-8091 Zürich, Switzerland; 4ICGM, Faculté Cochin-GDPM, 75014 Paris, France

The anaphase-promoting complex or cyclosome (APC/C) is an ubiquitin protein ligase that together with Cdc20 and Cdh1 targets mitotic proteins for degradation by the proteosome. APC–Cdc20 activity during mitosis triggers anaphase by destroying securin and cyclins. APC–Cdh1 promotes degradation of cyclins and other proteins during G1. We show that loss of APC/C during embryogenesis is early lethal before embryonic day E6.5 (E6.5). To investigate the role of APC/C in quiescent cells, we conditionally inactivated the subunit Apc2 in mice. Deletion of Apc2 in quiescent hepatocytes caused re-entry into the cell cycle and arrest in metaphase, resulting in liver failure. Re-entry into the cell cycle either occurred without any proliferative stimulus or could be easily induced. We demonstrate that the APC has an additional function to prevent hepatocytes from unscheduled re-entry into the cell cycle.

Keywords: Anaphase-promoting complex; APC; cell cycle; G0; quiescent cells

Supplemental material is available at http://www.genesdev.org.

Received September 15, 2003; revised version accepted November 10, 2003.

Ubiquitin-mediated proteolysis has crucial roles in a wide variety of biological processes, including mitosis, transcriptional control, regulation of the immune system, signal transduction, and development (Ben-Neriah 2002; Conaway et al. 2002; Harper et al. 2002; Peters 2002). The first step in this form of proteolysis is the covalent attachment of multiple ubiquitin chains to target proteins. This is accomplished by specific ubiquitin protein ligases, many of which have RING-H2-finger domains as part of their catalytic cores [Jackson et al. 2000; Pickart 2001].

One of the first RING-finger ubiquitin protein ligases to be associated with a defined and essential physiological function is the multisubunit anaphase-promoting complex or cyclosome (APC/C; Irniger et al. 1995; King et al. 1995; Sudakin et al. 1995). The APC/C consists of 11 subunits and is essential for destroying proteins during mitosis in all eukaryotic cells (Peters et al. 1996; Zachariae et al. 1996). Two of the APC/C’s subunits, Apc2 and Apc11, constitute a catalytic core, which is capable of ubiquitinating target proteins in vitro (Gmachl et al. 2000; Leverson et al. 2000). Apc2 and Apc11 interact with each other and contain cullin homology and RING-finger domains respectively [Yu et al. 1998; Zachariae et al. 1998b; Tang et al. 2001].

The finding that B-type cyclins are rapidly degraded by the APC/C during G1 phase raised the possibility that it

5Corresponding author.
E-MAIL k.nasmyth@nt.imp.univie.ac.at; FAX 0043-1-798-9390.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.285404.
has important functions also in nonmitotic cells (Amon et al. 1994; Brandeis and Hunt 1996; Ingrig and Nasmyth 1997). In yeast, the APC/C’s activity during G1 depends not on Cdc20 but on a related protein called Cdh1 (Visintin et al. 1997; Zachariae et al. 1998a). The activities of Cdc20 and Cdh1 are complementary. Whereas Cdc20 is active during mitosis, Cdh1 is active during G1, when Cdk1 is inactive. Phosphorylation of Cdh1 by Cdk1 prevents it from binding the APC/C for much of the cell cycle (Schwab et al. 2001). The activities of Cdk1 and APC–Cdh1 are therefore mutually exclusive. The activity of one causes inactivity of the other and vice versa. The functions of APC–Cdh1 during G1 are less well understood than those of APC–Cdc20 during mitosis. Inactivation of Cdh1 or the APC/C causes budding yeast cells to enter S phase in the presence of sex pheromones (Ingrig and Nasmyth 1997) and fission yeast cells to do so when starved of a nitrogen source (Kominami et al. 1998). Hyperactivity of Cdk1 due to accumulation of cyclins may be at least partly responsible for these phenomena.

The APC/C is also active in quiescent mammalian cells. Most if not all APC/C subunits as well as Cdh1–like proteins are found in tissues composed of fully differentiated cells (Gieffers et al. 1999). Indeed, a fully active complex can be isolated from adult mouse brain tissues. Mammals possess several different Cdh1 proteins, which have different tissue distributions and target specificities (Wan and Kirschner 2001). Loss of a Cdh1 homolog in chicken DT40 cells has little effect on cell proliferation but leads to accumulation of mitotic cyclins in G1, and abrogates G1 arrest mediated by rapamycin (Sudo et al. 2001). In Drosophila, Cdh1 (known as fzr) is required when cells exit from the mitotic cycles during embryogenesis and for the destruction of mitotic cyclins in postmitotic embryonic cells (Sigrist and Lehner 1997). The finding that Smad2 binds to Cdh1 and thereby promotes the APC/C’s ability to ubiquitinate SnoN, an inhibitor of TGF–β signaling, suggests that the APC–Cdh1 might facilitate the antiproliferative effects of TGF–β (Stroschein et al. 2001; Wan et al. 2001). However, the physiological significance of this process is not understood.

In this paper we address APC/C’s function in quiescent cells using a genetic approach. Instead of identifying potential substrates whose ubiquitination in quiescent cells may be of physiological importance, we generated a conditional allele of Apc2 that permitted us to inactivate this crucial APC/C subunit in nonproliferative tissues. We find that ablation frequently triggers the spontaneous entry into the cell cycle of quiescent hepatocytes. Livers lacking APC/C respond more readily to known proliferative signals.

Results

Conditional inactivation of the Apc2 allele

To identify possible orthologs or paralogs of the Apc2 protein, an NCBI–PSI-BLAST search for Apc2 was performed. A single mouse Apc2 ortholog (XP_130088.1) and no paralogs were found (Supplemental Fig. 1).

For inactivation of Apc2 in a conditional manner, exons 2–4 of the Apc2 locus were flanked by loxP sites (floxed allele). Deletion of exons 2–4 by Cre-mediated recombination leads to a frameshift with stop codons in all three open reading frames. Following transfection with the targeting vector, G418-resistant E14.1 ES cell clones in which one Apc2 allele had been replaced by the targeting construct were identified by Southern blotting. Transient transfection of correctly targeted clones with a plasmid that expressed the Flpc-recombinase created a floxed allele, whereas transfection with a plasmid expressing Cre-recombinase created a deletion allele lacking exons 2–4 (Δ allele). Two independent ES cell clones carrying floxed or deletion alleles of Apc2 were injected into C57Bl/6 blastocysts. Chimeras were crossed with C57Bl/6 mice to obtain germ-line transmission of either the Apc2 floxed or the Apc2Δ allele (Fig. 1A–E).

Disruption of Apc2 causes embryonic lethality

To determine if Apc2 is essential for mouse development heterozygous Apc2+/Apc2− mice were intercrossed. No homozygous Apc2+/Δ mice were born in a total of 120 live births from intercrosses between Apc2+/− mice. Apc2+/− mice showed no abnormalities and were born with the expected frequencies (Table 1). Embryos from Apc2+/− intercrosses were analyzed at E6.5 and E9.5. At E6.5 as well as at E9.5, no homozygous Apc2+/Δ embryos were found (Table 1). We conclude that Apc2 is required for early embryogenesis. Mice heterozygous for Apc2 floxed alleles as well as Δ alleles showed no abnormalities. Homozygous mice carrying Apc2 floxed alleles as well as Apc2+/− mice had no defects, indicating that the floxed allele of Apc2 is fully functional.

Loss of the APC/C in quiescent hepatocytes causes acute liver failure

To address APC/C’s function in differentiated cells, we investigated the consequences of deleting Apc2 in quiescent hepatocytes of adult livers. We used an Mx-Cre transgenic line in which Cre can be induced by injection of poly(I) poly(C) (pl/C, Kühn et al. 1995). Six-week-old mice heterozygous for the Mx-Cre transgene and carrying one floxed and one Δ allele of Apc2 were injected twice with pl/C. All Apc2+/−/floxed and Apc2Δ+/−/Mx-Cre mice were used as controls. Southern and Western blot analysis confirmed that pl/C caused efficient deletion of the Apc2 floxed allele (Fig. 2B,C).

Most Apc2+/−/Mx-Cre mice died during the second week after the first pl/C injection (Fig. 2A). Histological analyses of livers removed shortly before death revealed dramatic changes in size and but no difference in four out of 10 Apc2+/−/Mx-Cre mice. Hematoxylin/eosin-staining of abnormal livers showed that most hepatocytes were greatly enlarged, lacked nuclear membranes, and con-
tained condensed chromosomes (Fig. 2E). On squashes from Apc2/H9004/floxMx-Cre livers, 72% of mitotic hepatocytes were in a prometaphase-like state with condensed chromatin. The shape of individual chromosomes was ill defined (Fig. 2D). Animals with such changes are referred to as affected, whereas animals whose livers showed no changes are referred to as unaffected. Southern blotting demonstrated that there was no correlation between the incidence of liver defects and the efficiency of Apc2 deletion (Fig. 2B; data not shown). PAS staining showed that the abnormal hepatocytes of affected Apc2/H9004/floxMx-Cre mice also lacked glycogen (Fig. 4A below). Levels of the liver enzymes alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH), as well as the levels of bilirubin, were highly elevated in affected Apc2/H9004/floxMx-Cre mice, indicating functional impairment and damage of hepatocytes. These mice died from acute liver failure. In unaffected Apc2Δ/fox Mx-Cre mice as well as in Apc2Δ/fox mice, ALT, GLDH, and bilirubin were within the normal range (Supplemental Fig. 2).

Rapid and severe bone marrow aplasia in Apc2-deficient mice

Because Mx-Cre-mediated recombination also occurs in hematopoietic cells, we measured hemoglobin levels. The four mice with normal livers all suffered from severe anemia, with hemoglobin levels below 5 g/dL, which could therefore be the cause of their death. Severe anemia was also observed in two of the mice with abnormal livers. The severe anemia caused by pl/C in Apc2Δ/fox Mx-Cre mice is caused by deletion of Apc2 in bone marrow cells. To investigate this further, we used FACS analysis (data not shown) and cytospins to analyze hematopoietic cells following pl/C injection. Severe bone marrow aplasia usually developed within 4 d of pl/C injection. By day 4, the majority of nucleated cells, including erythroblasts, had disappeared from the bone marrow, which contained mainly erythrocytes and a few lymphocytes (Fig. 3A). On day 3 after pl/C injection, we observed an increase in the number of mitotic cells in Apc2Δ/fox Mx-Cre mice compared with the Apc2Δ/fox control mice. These findings indicate that abolition of the APC/C from hematopoietic cells leads to metaphase arrest followed by cell death. This process occurred very

<table>
<thead>
<tr>
<th>Table 1. Embryonic lethality in Apc2Δ/fox mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apc2Δ/fox × Apc2Δ/fox</td>
</tr>
<tr>
<td>E6.5</td>
</tr>
<tr>
<td>E9.5</td>
</tr>
<tr>
<td>p21</td>
</tr>
</tbody>
</table>

From intercrosses between Apc2Δ/fox mice, no Apc2Δ/fox mice were born. At embryonic days E6.5 and E9.5 all embryos were Apc2Δ/fox or heterozygous for the Apc2Δ allele.
rapidly so that we never observed a dramatic accumulation of mitotic cells. It is nevertheless unclear whether cell death arising from mitotic arrest can account for the rapid disappearance of bone marrow cells.

Metaphase arrest in unaffected Apc2-deficient hepatocytes after 2/3 hepatectomy

On Western blot analysis, Apc2 protein levels were reduced in affected as well as in unaffected livers, and no differences in protein levels were observed [Figs. 2C and 5E below]. To address whether any functional Apc2 protein persisted in the hepatocytes of mice whose livers appeared normal after deletion of their Apc2 gene, we investigated the consequences of a 2/3 hepatectomy, which induces cell cycle re-entry. To circumvent lethality due to anemia, 10 Apc2^{fl/fl} Mx-Cre mice and 10 Apc2^{fl/fl} mice had bone marrow transplants from Apc2^{+/+} littersmates. Then 2 x 400 µg/mouse pl/C were injected 4 wk posttransplantation, only after ensuring

Figure 2. Liver failure in Apc2^{fl/fl} Mx-Cre mice. [A] Survival curve of adult Apc2^{fl/fl} Mx-Cre and control mice (Apc2^{+/+} and Apc2^{+/+} Mx-Cre mice) after two injections of 400 µg of pl/C. Most mice died between day 11 and day 14 after the first pl/C injection. (B) Southern blot analysis of Apc2^{fl/fl} or Apc2^{fl/fl} Mx-Cre livers; (np) no phenotype, (p) with phenotype. (C) Western blot analysis of Apc2^{fl/fl} and Apc2^{fl/fl} Mx-Cre livers after pl/C injection for Apc2-protein levels. Actin is the loading control; (np) no phenotype, (p) phenotype. (D) Squashes from Apc2^{fl/fl} Mx-Cre livers. Seventy-three percent of mitotic cells were in a prometaphase-like state with condensed chromosomes. (E) Hematoxylin/eosin staining of livers from Apc2^{fl/fl} and Apc2^{fl/fl} Mx-Cre mice. Apc2^{fl/fl} Mx-Cre hepatocytes appeared larger and had condensed chromosomes.}

Figure 3. 2/3 hepatectomy in transplanted Apc2^{fl/fl} Mx-Cre mice causes metaphase arrest. [A] Cytospins of bone marrow cells at day 3 and day 4 after 1 x 400 µg pl/C injection. In the Apc2^{fl/fl} Mx-Cre bone marrow, the number of mitotic cells (arrowheads) was increased at day 3 after pl/C. At day 4, only erythrocytes and a few mature lymphocytes could be detected in Apc2^{fl/fl} Mx-Cre bone marrow. [B] Hematoxylin/eosin staining of Apc2^{fl/fl} and Apc2^{fl/fl} Mx-Cre livers 72 h after 2/3 hepatectomy in unaffected Apc2^{fl/fl} Mx-Cre mice. In the Apc2^{fl/fl} Mx-Cre mice, 70% of the hepatocytes were enlarged and 50% of the cells were in metaphase. (C) Squashes from Apc2^{+/+} and Apc2^{fl/fl} Mx-Cre livers 48 h after 2/3 hepatectomy. (a–g) Mitosis in Apc2^{+/+} hepatocytes. (a) Prophase; (b) early prometaphase, NEB but no orientation of chromosomes; (c) late prometaphase, not all chromosomes are aligned yet at the equatorial plate; (d) metaphase, sideview, all chromosomes were aligned at the equatorial plate; (e) anaphase, sister chromatids were segregating to opposite poles; (f) telophase; (g) early G1. (h–l) Mitosis in Apc2^{fl/fl} Mx-Cre hepatocytes. (h) Prophase, no difference from Apc2^{+/+} hepatocytes; (i) prometaphase, the shape of individual chromosomes appeared ill defined; (j) prometaphase-like cell with condensed chromatin; (k) metaphase, polar view; (l) metaphase-like cell with condensed chromatin, but individual chromosomes could not be observed.

GENES & DEVELOPMENT
that blood counts were normal. Three \textit{Apc2}\textsuperscript{Δ/floxFlox}\textit{Mx-Cre} but no \textit{Apc2}\textsuperscript{Δ/floxFlox}\textit{Mx-Cre} mice died of liver failure over a period of 8 wk after \textit{pI/C} injection [data not shown]. In the seven surviving \textit{Apc2}\textsuperscript{Δ/floxFlox}\textit{Mx-Cre} mice, 2/3 hepatectomy caused death within 3–5 d. Histological analyses showed that 70% of the mitotic hepatocytes of these mice were arrested in metaphase [Fig. 3B], despite appearing normal before the operation [data not shown]. Squashes from \textit{Apc2}\textsuperscript{Δ/floxFlox}\textit{Mx-Cre} livers confirmed that their chromosomes were condensed and frequently aligned in a manner similar to metaphases from \textit{Apc2}\textsuperscript{+/+} mice. However, the chromosomes of cells in metaphase lacking Apc2 were overcondensed and the metaphase plates were smaller than in the controls in 95% of the cells. [Fig. 3C, k and l]. \textit{Apc2}\textsuperscript{Δ/foxx} mice survived this regime, and their hepatocytes appeared no different from those of wild-type mice. These findings imply that the Apc2 protein really had been removed from unaffected livers whose Apc2 gene had been deleted. They also demonstrate that the APC/C is essential for the onset of anaphase in mammalian cells.

\textit{Analysis of APC/C substrates in Apc2-deficient hepatocytes}

We next characterized the cell cycle state of hepatocytes from livers that were clearly affected merely by deletion of Apc2 in more detail. Very few \textit{Apc2}\textsuperscript{Δ/foxx} (<2%) but 50%–70% of \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} hepatocytes were Ki67 and pH3 positive [Fig. 4B]. In situ immunofluorescence [Fig. 5A–D] showed that a high percentage of the cells were positive for Ki67, pH3, Cdc20, and cyclin A2. In mitotic cells, both cyclin A2 and Cdc20 were associated with the spindle as well as distributed throughout the cytoplasm owing to nuclear envelope breakdown [NEB]. The affected livers also contained hepatocytes that had not yet entered mitosis but nevertheless contained high levels of cyclin A2 within their nuclei. Western blotting [Fig. 5E] showed that deletion of Apc2 greatly reduced the amount of Apc2 protein in both affected and unaffected livers. Affected but not unaffected livers had high levels of proteins known to be APC/C substrates. The levels of the G1-substrates cyclin D1 and Snx20 as well as the mitotic substrates cyclin A2, cyclin B1, securin, Plk1, and Cdc20 were comparable to those of mouse embryonic fibroblasts [MEF] treated with nocodazole. The cyclin kinase inhibitor p27 was down-regulated in affected livers. Livers apparently unaffected by deletion of Apc2 had low levels of APC/C targets and high levels of p27. Cyclin E and Cdc6 were expressed in hepatocytes from affected and unaffected \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} mice as well as from \textit{Apc2}\textsuperscript{Δ/foxx} mice, and there was no difference in the expression levels [data not shown]. These results suggest that deletion of Apc2 by \textit{Mx-Cre} induces hepatocytes of some but not all livers to enter into a proliferative state, which results in a mitotic arrest. The cell cycle re-entry of hepatocytes from \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} mice cannot be caused by DNA damage induced by extended expression of the Cre recombinase [Loonstra et al. 2001; Silver and Livingston 2001] because \textit{pI/C} caused no significant increase in the number of Ki67-positive hepatocytes in \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} mice. Fewer than 3% of hepatocytes from \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} or \textit{Apc2}\textsuperscript{Δ/foxx} livers were Ki67-positive at day 3, 7, and 28 after \textit{pI/C} injection. [Supplemental Fig. 4].

\textit{Deletion of Apc2 in hepatocytes alone causes their proliferation and mitotic arrest}

The finding that deletion of Apc2 by \textit{Mx-Cre} causes hepatocyte proliferation suggests that APC/C activity may be necessary to maintain hepatocytes in a quiescent state. This would be a novel activity for a ubiquitin ligase hitherto implicated in promoting anaphase. The finding that deletion of Apc2 by \textit{Mx-Cre} causes an increase in the number of Ki67-positive hepatocytes in \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} mice. Fewer than 3% of hepatocytes from \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} or \textit{Apc2}\textsuperscript{Δ/foxx} livers were Ki67-positive at day 3, 7, and 28 after \textit{pI/C} injection. [Supplemental Fig. 4].

The finding that deletion of Apc2 by \textit{Mx-Cre} causes hepatocyte proliferation suggests that APC/C activity may be necessary to maintain hepatocytes in a quiescent state. This would be a novel activity for a ubiquitin ligase hitherto implicated in promoting anaphase. However, our observation that Apc2 deletion causes anemia as well as liver failure raises the possibility that the proliferation of hepatocytes might be a secondary effect of apoptosis. To address this, we analyzed livers from \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} and \textit{Apc2}\textsuperscript{Δ/foxx} mice at day 0, 1, 2, 3, 4, and 5 after \textit{pI/C} injection by TUNEL assay. In \textit{Apc2}\textsuperscript{Δ/foxx}\textit{Mx-Cre} mice, anemia occurs within 3–4 d after \textit{pI/C} injection. Cre-mediated Apc2 deletion caused no increase in TUNEL positive cells [Supplemental Fig. 3A]. We did,
however, find 6% of TUNEL positive cells in affected \(Apc^{2\text{flox}}/\text{H9004/floxMx-Cre}\) livers shortly before death (Supplemental Fig. 3B). These findings are inconsistent with the notion that apoptosis might be a proliferative stimulus in \(Apc^{2\text{flox}}/\text{H9004/floxMx-Cre}\) mice.

To further address whether hypoxia was the cause for re-entry of quiescent hepatocytes into the cell cycle, we investigated the effect of deleting \(Apc2\) solely in hepatocytes using a \(TTR\)-Cre transgene. In \(TTR\)-Cre transgenic mice, the Cre-recombinase expressed from the \(TTR\) promoter is flanked by two hormone-binding domains of the murine estrogen receptor. This form of Cre remains inactive until induced by injections of 4-hydroxytamoxifen (4-OHT). Addition of 4-OHT to adult mice carrying the \(TTR\)-Cre transgene therefore causes Cre-mediated recombination exclusively in hepatocytes [Tannour-Louet et al. 2002]. Southern and Western blots showed that 10 daily 4-OHT injections in \(Apc^{2\text{flox}}/\text{H9004/floxMx-Cre}\) mice caused efficient deletion of \(Apc2\) from livers as well as depletion of the \(Apc2\) protein. 4-OHT caused death associated with liver failure [usually within 3 wk of the first injection] in 40% of \(Apc^{2\text{flox}}/\text{H9004/floxMx-Cre}\) mice (Fig. 6A). Hepatocytes at the time of death had the same appearance as those produced by deletion of the APC/C by \(Mx\)-Cre, namely, they were greatly enlarged and arrested in metaphase (Fig. 6D). Because \(TTR\)-Cre does not delete in fibroblasts of \(Apc^{2\text{flox}}/\text{H9004/floxMx-Cre}\) livers, we observed proliferation of fibroblasts as a response to the increase of hepatocyte cell death (Fig. 6D) and residual bands on Southern and Western blots (Fig. 6B,C). Because no bone marrow aplasia occurred in these mice (data not shown), hypoxia can be excluded as a proliferation stimulus. We conclude that deletion of \(Apc2\) in hepatocytes alone is sufficient to trigger their entry into the cell cycle in 40% of the mice. In 60% of \(Apc^{2\text{flox}}/\text{H9004/floxMx-Cre}\) mice, 4-OHT had no effect despite comparable levels of \(Apc2\) deletion (Fig. 6B,C). Furthermore, the histology of livers of these unaffected mice looked normal (data not shown).
A low proliferation stimulus is sufficient to induce re-entry into the cell cycle in unaffected Apc2-deficient hepatocytes

The finding that Apc2 deletion frequently causes resting hepatocytes to enter the cell cycle suggests that the APC/C has a key role in restricting cell cycle entry. However, in half of the Apc2-deficient mice, hepatocytes remain quiescent. One explanation for this phenotypic variation is that abolition of APC/C lowers the “threshold” for cell cycle entry. In some mice, endogenous mitogenic signals are now capable of triggering cell cycle entry, but in others they are not.

This idea predicts that asymptomatic Apc2-deficient hepatocytes should be more readily stimulated to enter the cell cycle by mitogenic signals, which sometimes causes them to enter the cell cycle without any obvious external stimulus.
Discussion

In addition to its key regulatory function during cell proliferation, the APC/C is known to be also active in post-mitotic differentiated cells (Gieffers et al. 1999). However, the significance of this activity is not understood. To investigate the role of the APC/C in quiescent cells, we conditionally deleted one of its subunits, Apc2, in hepatocytes in vivo.

The mammalian APC/C is needed for anaphase

Genetic studies have implicated the APC/C in triggering anaphase in a wide variety of eukaryotic organisms. Mutations that affect the APC/C or its activator Cdc20 cause yeast (Irniger et al. 1995), fly (Sigrist and Lehner 1997), and worm (Furuta et al. 2000; Golden et al. 2000) cells to arrest in a metaphase-like state with unseparated sister chromatids. Injection of antibodies specific for CDC16/APC6 and CDC27/APC3 produce a similar phenotype in mammalian tissue culture cells (Tugendreich et al. 1995). Nevertheless, antibody injection experiments are difficult to interpret, and the effects of inactivating the APC/C in mammalian cells has never been investigated using genetics. Because the consequences of inactivating genes in the mouse have often proven different from that expected from tissue culture experiments, it was important to establish whether the APC/C is essential for mitosis in living animals.

Our inability to detect Apc2<sup>+/−</sup> E6.5 embryos arising from crosses between Apc2<sup>+/−</sup> mice implies that the APC/C is essential for embryonic development. This is consistent with the finding that inactivation of the APC/C subunit Apc10 in mice is early embryonic lethal (Pravtcheva and Wise 1996). Using the Mx-Cre system, we managed to delete Apc2 in resting hepatocytes. 2/3 hepatectomy caused these Apc2-deficient although otherwise apparently normal quiescent cells to embark on proliferation. Our finding that the majority of these cells arrested in a metaphase-like state 3 to 5 d after 2/3 hepatectomy demonstrates that the APC/C is necessary for anaphase in mammals in vivo.

Loss of the APC/C in hepatocytes leads to cell cycle re-entry

Abolition of the APC/C has also dramatic consequences in quiescent hepatocytes. Within 2 wk of Apc2 deletion caused by pl/C stimulation of an Mx-Cre transgene, most mice died. In some cases, this appears to have been caused by severe anemia due to loss of APC/C in the bone marrow. In most cases, however, death appears to be caused by liver failure brought about by the arrest of hepatocytes in a mitotic state during which normal liver function cannot be maintained. Crucially, deletion of Apc2 solely in hepatocytes [using a 4-hydroxytamoxifen-inducible TTR-Cre transgene] produces a similar phenotype, which cannot therefore be a secondary consequence of bone marrow failure. It is still unclear whether the massive changes brought about by deletion of Apc2 in the bone marrow are caused by apoptosis arising from mitotic arrest or by some more direct effect on cell survival. The liver pathology is caused by the spontaneous entry of a large number of hepatocytes into the cell cycle, which in the absence of APC/C causes their arrest in a mitotic-like state. Several known substrates of the APC/C such as mitotic cyclins, securin, and SnoN accumulate to high levels in these cells, whereas other S phase-promoting proteins like Cdc6 and cyclin E are expressed at the same level as in wild-type hepatocytes. This finding implies that the APC/C has a crucial role in quiescent hepatocytes, namely, to restrain their re-entry into the cell cycle. The APC/C could perform this function either by suppressing the production of extracellular mitogens or more directly by suppressing accumulation of intracellular proteins capable of promoting proliferative growth.

Surprisingly, deletion of Apc2 fails to cause a proliferative response in about half of all mice. Despite the disappearance of Apc2 protein, the hepatocytes of such mice remain in a quiescent state that appears very similar to that of wild-type. These asymptomatic Apc2-deficient hepatocytes are nevertheless in an altered state because they are more readily stimulated to embark on DNA replication than wild-type. Within 48 h, 1/4 hepatocytes caused many Apc2-deficient hepatocytes to become Ki67<sup>+</sup> and to enter S phase. We conclude that abolition of the APC/C causes a major change in the state of quiescent hepatocytes such that they are more readily stimulated to embark on cell proliferation. Their readiness to enter the cell cycle allows them to do so either spontaneously or in response to mitogenic signals. Necrosis or apoptosis can be excluded as a stimulus for re-entry into the cell cycle because depletion of the APC/C also caused hepatocytes of livers apparently unaffected by Apc2 deletion to enter the cell cycle upon 1/4 hepatectomy. A failure to destroy SnoN seems unlikely to be responsible because SnoN levels, although clearly elevated in APC/C-deficient cells that have arrested in a mitotic state, are not significantly increased in asymptomatic APC/C-deficient livers. We also found no obvious changes in the levels of other APC/C substrates such as cyclin B1, securin, Plk, and Cdc20, and only a very small if any increase in the level of cyclin A2.

Although no other study has directly addressed the APC/C’s role in quiescent cells, our finding that abolition of the APC/C promotes cell cycle entry is consistent with the finding that Drosophila embryos lacking fizzy-related (Cdh1) progress through an extra cell cycle (Sigrist and Lehner 1997) and with the finding that chicken tissue culture cells lacking a particular Cdh1 gene are less likely to be arrested in G<sub>1</sub> by rapamycin (Sudo et al. 2001). Both these and our data all point to a key role for the APC/C in suppressing proliferation as well as promoting anaphase during mitosis.

Materials and methods

NCBI-PSI-BLAST search for Apc2.

Proteins from Homo sapiens, Mus musculus, Drosophila melanogaster, Caenorhabditis elegans, Schizosaccharomyces

GENES & DEVELOPMENT
Homogenization of tissues was performed in extraction buffer 1:1 Tween 20:NP-40 buffer. Tween 20 buffer: 50 mM HEPES at pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.2% Tween 20. NP-40 buffer: 1% NP-40, 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10 mM EDTA, supplemented with phosphatase and protease inhibitors. Then 100 µg protein per lane was loaded on a 8% and 13% gel and transferred to a PVDF membrane (Millipore). For detection of proteins antibodies against cyclin B1 [1:1000; Upstate Biotechnology], cyclin A2 [1:1000; gift from Mark Carrington, University of Cambridge, Cambridge, UK], Apc2 and Cdc20 [1:1000; gifts from Christian Gieffers, Affims, Wien, Austria, and Edgar Kramer, MPI of Neurobiology, Martinsried, Germany], SnoN [5 µg/mL Cascade Bioscience], cyclin D1, PIK (1:250; Zymed), and p27 [1:100; Santa Cruz], actin (1:100; Sigma), and tubulin (1:1000, Sigma) were used.

**Immunofluorescence**

Tissues were frozen in liquid nitrogen and embedded in OCT tissue TEC. Then 10-µm cryosections were cut and fixed in 4% paraformaldehyde for 20 min. Sections were incubated in 50 mM NH₄Cl in PBS and 0.1% Triton-X for 3 min. Unspecific binding was blocked with 3% BSA in PBST for 1 h. For dilution of primary and secondary antibody, blocking solution was used and sections were incubated for 1 h. After three washes in PBST, slides were stained with 1 µg/mL DAPI and mounted (Vectashield H-1000, Vector Laboratories). For Apc2 staining, tissues were fixed in 4% paraformaldehyde for 20 min and incubated in 0.25% Triton-X for 10 min. Primary antibodies against cyclin A2 (gift from Mark Carrington) and Cdc20 (gift from Edgar Kramer) were used in a concentration of 1:50. For antibodies against tubulin (Sigma), pH3 [Upstate Biotechnology], and Ki67 [Novocastra Laboratories], the concentration was 1:1000 and against Apc2 1:250 (gift from Christian Gieffers). Secondary antibodies were anti-mouse- (1:1000) or anti-rabbit-Alexa 488 1:400 (Molecular Probes).

**Immunohistochemistry and TUNEL assay**

Tissues were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. Then 5-µm paraffin sections were cut, rehydrated, and boiled in 10 mM NaCitrat (pH 6) for 13 min. Unspecific binding was reduced by incubation in 0.5% H₂O₂ for 15 min. After blocking in 3% BSA/PBS, sections were stained with anti-Ki67 (1:1000, Novocastra Laboratories) or anti-pH3-antibody [1:1000, Upstate Biotechnology] overnight. Secondary antibody incubation and enzymatic reaction were performed by using the ABC and DAB staining kit (Vector Laboratories). Sections were counterstained with hematoxylin for 3 min.

For the detection of apoptotic cells the In Situ Cell Death Detection Kit {Roche} was used on 0.1% NaCitrat and 0.1% Triton-X pretreated cryosections.

**Cytopsins**

For cytopsins, 5 × 10⁵ cells/well were centrifuged on a slide at 700 rpm for 7 min. Cells were stained with Quick stain [Merck].

**Squashing procedure**

Livers were cut into small [1–3 mm³] pieces and immediately fixed in freshly prepared Carnoy’s (methanol:acetic acid). After five changes of fixative, the material was stored at −20°C. For cytological preparations, small pieces were put on a slide, and several drops of 50% acetic acid in water were added to soften the tissue. When the tissue lost its whitish color (~30 sec), most of the liquid was removed using filter paper. Next, three
drops of lactopropionic orcein (2% of orcein-Gurr in 1:1 lactic acid:propionic acid) was added for 2–3 min. A coverslip was placed on top, and the material was gently dispersed by smoothly moving the coverslip with the tip of a pencil. After 1 h of staining, one corner of the coverslip was held with a filter paper to avoid movement and a spiral was drawn on the coverslip with the pencil starting from the center of the material to the edges. A piece of filter paper was put onto the coverslip, and the material was strongly squashed.

**BrdU staining**

For BrdU staining, 50 µg/g BrdU was injected with the anesthetic for 2/3 hepatectomy i.p. and repeated every 24 h. Livers were fixed in 4% paraformaldehyde overnight and dehydrated. After embedding into paraffin, 6-µm sections were cut. After rehydration, sections were incubated in 2 N HCl for 45 min, 1× Trypsin for 45 min, and 0.5% H2O2 for 30 min. Unspecific binding was blocked by incubation in 3% BSA in PBS, and sections were incubated with anti-BrdU-antibody (1:100; Zymed) overnight. Sections were blocked by incubation in 3% BSA in PBS, and sections were counterstained with hematoxylin for 3 min.

**Induction of Cre in vivo**

For expression of Mx-Cre, 400 µg (1 mg/mL) of pI/C (Pharmacia) i.p. was injected twice with an interval of 72 h. For deletion in expression of Mx-Cre, 400 µg (1 mg/mL) of pI/C (Pharmacia) i.p. was used.

**Bone marrow transplantation**

Apc2+/lox Mx-Cre and Apc2+/lox mice were irradiated with 1100 rad. Then, 24 h after irradiation, 4 × 10⁶ bone marrow cells from Apc2+/+ donor littersmates were injected i.v. Successful bone marrow reconstitution was evaluated 4 wk after transplantation by blood checks. A total of 2 × 400 µg/mouse pI/C i.p. with an interval of 72 h was injected.

**2/3 and 1/4 hepatectomy**

Mice were anesthetized with 13 µL/g 2.5% Avertin i.p., and an abdominal transversal incision below the xyphoid process was performed. For 2/3 hepatectomies, the large left and the two median lobes were ligated and removed. For 1/4 hepatectomies, the left lobe was taken out. In Sham-operated animals, the large left and the two median lobes were exposed and put back without any removal of tissue.

**Acknowledgments**

We thank Hartmut Beug, Meinrad Busslinger, Jan-Michael Peters, Erwin Wagner, and their groups for providing advice and reagents. Special thanks go to Donal O’Carroll for the introduction to ES cell work and Hans Christian Theussl for blastocyst injections. Christian Gieffers, Edgar Kramer, and Mark Carrington provided antibodies, and Francis Stewart the Flpe-plasmid.

We received Cre-transgenic mice from Wolfgang Wurst with permission from Ralf Kühn, Jamey Marth, Mireille Vasseur-Cognet, and the Busslinger and Wagner group. We thank Nicole Fink for isolation of embryos at E6.5 and Grzegorz Sumara for help with hepatectomies. Alexander Schleifer performed the bioinformatics searches. We appreciate the critical reading and discussion of the manuscript by Hartmut Beug and Jan-Michael Peters. The IMP is funded by Boehringer Ingelheim, and this work was partly supported by a network grant from the European Community for R.R. (contact number: QLG1-CT-2001-02026, shared costs action U2P2), a Marie Curie fellowship from the European Community for R.R., and a Research Fellowship from the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for R.G.W.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

**References**


site-specific mutagenesis in the basal layer of the epidermis: Comparison of the recombine activity of the tamoxifen-inducible CRE-ER(T) and CRE-ER(T2) recombinases. Nucl. Acids Res. 27: 4324–4327.


Kominami, K., Seth-Smith, H., and Toda, T. 1998. Apc10 and Ste9/Srl1, two regulators of the APC-cyclosome, as well as the CDC915r Kirsten Ruml are required for G1 cell-cycle arrest in fission yeast. EMBO J. 17: 5388–5399.


Tugendreich, S., Tomkiel, J., Earnshaw, W., and Hieter, P. 1995. CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. Cell 81: 261–268.


