Liver tetraploidization is controlled by a new process of incomplete cytokinesis

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Summary
Cytokinesis is precisely controlled in both time and space to ensure equal distribution of the genetic material between daughter cells. Incomplete cytokinesis can be associated with developmental or pathological cell division programs leading to tetraploid progenies. In this study we decipher a new mechanism of incomplete cytokinesis taking place in hepatocytes during post-natal liver growth. This process is initiated in vivo after weaning and is associated with an absence of anaphase cell elongation. In this process, formation of a functional contractile actomyosin ring was never observed; indeed, actin filaments spread out along the cortex were not concentrated to the putative site of furrowing. Recruitment of myosin II to the cortex, controlled by Rho-kinase, was impaired. Astral microtubules failed to contact the equatorial cortex and to deliver their molecular signal, preventing activation of the RhoA pathway. These findings reveal a new developmental cell division program in the liver that prevents cleavage-plane specification.

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Introduction
Cell division ends with the processes of nuclear division (karyogenesis) and cytosolic separation (cytokinesis). In a typical animal mitosis, coordinated actions from astral and central spindle microtubules to the actin cytoskeleton and membrane systems are essential for proper cytokinesis (Eggert et al., 2006; Glotzer, 2001). Variations of the cytokinesis process are observed in certain biological contexts. Incomplete cytokinesis very likely contributes to cancer progression, with common solid tumours tending to exhibit tetraploid cells (Gupta, 2000). During postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization during which hepatocytes of several ploidy classes emerge as a result of modified cell-division cycles. This process generates the successive appearance of tetraploid and octoploid cell classes with one or two nuclei. Thus, in the liver of a newborn rat, hepatocytes are exclusively diploid (2n). In adult rats, approximately 25% of hepatocytes are diploid, 70% are tetraploid (binucleated 2×2n or mononucleated 4n) and 5% are octoploid (binucleated 2×4n or mononucleated 8n). The degree of polyploidization varies in different mammals. In humans, the number of polyploid cells averages 30-40% in the adult liver (Kudryavtsev et al., 1993; Toyoda et al., 2005). In adults, liver polyploidization is differentially regulated upon loss of liver mass and liver damage. Interestingly, partial hepatectomy induces marked cell proliferation followed by an increase in liver ploidy (Sigal et al., 1999). By contrast, during hepatocarcinoma (HCC), growth shifts to a non-polyploidizing pattern and expansion of the diploid hepatocyte population is observed in neoplastic nodules (Seglen, 1997). We previously uncovered the sequential appearance during liver growth of binucleated 2×2n and mononucleated 4n hepatocytes from a diploid hepatocyte population (Guidotti et al., 2003). Furthermore, we showed in vitro that binucleated 2×2n hepatocytes emerge as a result of...
an incomplete cytokinesis. These tetraploid cells are capable of proliferation. In fact, binucleated hepatocytes are able to proceed through S phase and the formation of a bipolar spindle during mitosis constituted the key step leading to the genesis of two mononucleated 4n hepatocytes (Guidotti et al., 2003).

In the current study, we investigated when the incomplete cytokinesis process is taking place during postnatal liver growth and how this specific division program is controlled. We deciphered, in the liver, a new physiological process of incomplete cytokinesis triggered by weaning. Mitotic hepatocytes achieved karyogenesis without establishing the cleavage plane because of the deficiencies of actin cytoskeleton and microtubule reorganization.

Results
In the liver, the event of incomplete cytokinesis is triggered by weaning
We set out to unveil when the incomplete cytokinesis process is initiated during postnatal liver growth. Mitotic events were detected in vivo by a simultaneous nuclear, microtubule and plasma membrane labelling on liver tissue sections. In suckling rats, all late-telophase hepatocytes presented a midbody and a cell shape characteristic of cleavage furrow ingression (Fig. 1A, left panel). In fed rats, we found that although some late-telophase hepatocytes were engaged in a normal cytokinesis process others presented a round shape with no midbody, indicating an absence of ingression (Fig. 1A, right panels). We analyzed whether this phenotype was correlated with weaning. From day 10 to day 25, we analyzed cytokinesis events; rats were weaned at 19 days after birth. We clearly observed that the proportion of incomplete cytokinesis events increased significantly 2 days after weaning (21-day-old rats, 10±1.3%), being maximal in 25-day-old rats (40±1.5%) (Fig. 1B). We next investigated why, after weaning, some hepatocytes completed cytokinesis and others did not. Based on the location of the blood vessels, hepatocytes of each liver lobule are divided into two subpopulations: an upstream periportal (PP; in contact with afferent blood) and a downstream perivenous (PV; surrounding the centrolobular efferent vein) population. Hepatocytes located in the PP and PV zones of the liver lobule

Fig. 1. An incomplete mode of cytokinesis takes place in the liver, triggered by weaning. (A) Immunostaining for β-catenin (red) and β-tubulin (green) of liver sections (10- and 25-day-old rats). Images of telophase were visualized taking into account condensed chromatin staining (Hoechst 33342, blue). Immunostaining allowed us to distinguish between telophase that completed or did not complete cytokinesis. The percentages of cells with complete cytokinesis are indicated. Bars, 3 μm. (B) Events of incomplete cytokinesis before and after weaning (fixed at 19 days, arrow). At each point, four rats were independently analyzed. Percentages of complete and incomplete cytokinesis in telophase cells were calculated using β-catenin/β-tubulin/Hoechst immunostaining (see A). A total of 50 telophase cells were analyzed per animal. Bars represent s.e., P<0.0001.

Fig. 2. Events of incomplete cytokinesis occur both in periportal (PP) and perivenous (PV) hepatocytes. (A) Immunostaining for glutamine synthetase (GS; proximal PV hepatocyte staining, green, right panel), Pepck1 (proximal PP hepatocyte staining, green, left panel) and β-catenin (membrane labelling, red, both panels) was performed on rat liver sections (25-day-old rats). β-catenin/Hoechst staining allowed us to distinguish between telophase that completed cytokinesis (progression of the membrane) and telophase that did not complete cytokinesis (no progression of the membrane) (Hoechst staining in blue). Bars, 40 μm. (B) Percentage of telophase cells that completed cytokinesis or did not were calculated in each region (proximal PP: GS-positive staining; proximal PV: Pepck1-positive staining; distal PP/PV: GS/Pepck1-negative staining). A total of 50 images of telophase were analyzed per animals (n=4). Bars represent s.e., P<0.0001.
show remarkable differences in the levels and activities of various enzymes and other proteins (Jungermann and Kietzmann, 1996). By complementary immunolocalizations of PP and PV hepatocytes, we defined the distribution of events of complete/incomplete cytokinesis throughout the entire lobule. We first confirmed the presence of a proliferation gradient decreasing from the PP to PV regions (Fig. 2), as previously described (Gebhardt and Jonitza, 1991). However, we detected incomplete cytokinesis events both in the PP and PV regions (Fig. 2), correlated with a similar proportion of binucleated hepatocytes in the two areas (data not shown). These results represent the first demonstration that an incomplete mode of cytokinesis takes place in the liver, initiated after weaning, with no zonation inside the hepatic lobule.

Incomplect cytokinesis is defined by an absence of anaphase cell elongation
To further investigate the precise mechanism that gives rise to tetraploid progenies, we cultured primary hepatocytes from rat before and after weaning and analyzed them by live-cell video microscopy. Under these conditions, hepatocytes divided just once and a maximum mitotic index of 10% is reached (Guidotti et al., 2003). Before weaning, time-lapse observations revealed that all diploid hepatocytes progressed normally through mitosis and gave rise solely to diploid progenies. As anaphase proceeded, cells elongated preceding furrow formation and ingression (Fig. 3A and supplementary material Movie 1). We next monitored hepatocyte division after weaning. Mitotic hepatocytes did not complete cytokinesis in 10±1.7% of cases (n=50) when isolated from 21-day-old rats and in 28±3.6% of cases (n=50) when isolated from 25-day-old rats. Hepatocytes undergoing incomplete cytokinesis did not exhibit dynamic shape changes; there was no evidence of furrow ingression (Fig. 3A and supplementary material Movie 2). To expand this finding, we measured cell elongation from metaphase to telophase (pole-to-pole distance) on hepatocytes isolated from 25-day-old rats. Elongation was greatly impaired during incomplete cytokinesis, being fourfold lower as compared with complete cytokinesis (Fig. 3B). In association with this defect, DNA-to-cortex distance decreased (Fig. 3C); in fact, the DNA masses crushed on the cortical polar region from late anaphase to early telophase (supplementary material Movie 2). We confirmed the defect in cell elongation in vivo using β-catenin staining (as shown in Fig. 1A). All hepatocytes in metaphase had the same shape, representative of the cell rounding process (length: 21.4±0.43 μm, width: 25.03±0.73 μm, n=50). In telophase, hepatocytes that completed cytokinesis had an elongated shape (length: 31±0.88 μm, width: 16.34±0.53 μm, n=50), whereas hepatocytes that did not complete cytokinesis kept the same shape as in metaphase (length: 22.51±0.75 μm, width: 27.54±0.91 μm, n=50). We therefore concluded that the physiological process of incomplete cytokinesis is associated with the absence of cell elongation.

Deficiency in actin cytoskeleton reorganization in cells that did not complete cytokinesis
To further investigate the cell elongation defect, we analyzed cytoskeleton rearrangements, which are crucial in determining the placement of the cleavage furrow (Murthy and Wadsworth, 2005; Yumura, 2001). We examined how remodelling of F-actin was coordinated during cytokinesis. When hepatocytes completed cytokinesis, the presence of an actin belt parallel to the cleavage plane was observed in early telophase (Fig. 4A, left panels). By contrast, during the incomplete cytokinesis process, this structure was always absent (Fig. 4A, right panels).
panels). We quantified pole-to-pole distribution of F-actin along the cortex. We showed that, when hepatocytes completed cytokinesis, F-actin concentrated to the equatorial cortex, demonstrating redistribution of the protein during anaphase-to-telophase transition (Fig. 4B). By contrast, F-actin uniformly localized all along the cortex during the process of incomplete cytokinesis (Fig. 4B). Because latrunculin A (LatA) prevents actin polymerization (Wakatsuki et al., 2001), we analyzed, after weaning, the effect of LatA treatment on the cell elongation defect. As described before, 30% of hepatocytes isolated from 25-day-old rats did not exhibit dynamic shape changes; in the presence of LatA, all hepatocytes elongated (supplementary material Fig. S1). This result clearly suggests that, during the incomplete cytokinesis process, cell elongation is impaired because of an absence of actin cytoskeleton reorganization. In Drosophila cells, Rho kinase (ROCK) is essential for anaphase cell elongation (Dean and Spudich, 2006; Hickson et al., 2006). In our system, we determined whether inhibition of ROCK had an effect on cell shape during anaphase on primary cultures isolated from rats before weaning. Remarkably, treatment of hepatocytes with a specific ROCK inhibitor resulted in a defect in cell elongation during cytokinesis (supplementary material Movie 3). By measuring cell elongation between metaphase and telophase, we clearly reproduced the same defect that we observed after weaning (Fig. 4C). ROCK is required for normal myosin II recruitment to the equatorial cortex (Dean et al., 2005; Hickson et al., 2006; Straight et al., 2003). Equatorial myosin II accumulation, thereafter, derives turnover of actin filaments along the equator to allow ingression to take place (Guha et al., 2005; Murthy and Wadsworth, 2005). We analyzed the localization of myosin II at the equatorial cortex before and after weaning. If hepatocytes completed cytokinesis, we always observed that, during anaphase, the phospho-regulatory light chain (phospho-RLC) of myosin II accumulated to the equatorial cortex (Fig. 4D and supplementary material Fig. S2). We conclude that the incomplete cytokinesis process is characterized by the absence of actin cytoskeleton rearrangement.

Astral microtubules failed to contact the equatorial cortex during the incomplete cytokinesis process

Mitotic spindle microtubules deliver spatially restricted signals to the cortex to promote furrowing and this occurs together with the signalling pathway that regulates equatorial cortical activity (D’Avino et al., 2005). Astral and central spindle microtubules are considered to be essential for cleavage-plane specification (Alsop and Zhang, 2003; Bringmann and Hyman, 2005; Canman et al., 2003; Inoue et al., 2004). We therefore examined the behaviour of microtubules in our model. Before weaning, as hepatocytes proceeded through anaphase, staining for β-tubulin revealed the presence of anti-parallel microtubules (the central spindle) and microtubules towards the cell cortex in the furrow and polar regions (Fig. 5A). In telophase, microtubules were compressed in the midzone as a consequence of furrow ingression (Fig. 5A). After weaning, organization of the microtubules network was identical in all hepatocytes until early anaphase (data not shown). However, in late anaphase, we clearly noticed that 30% of hepatocytes presented a disrupted central spindle and astral equatorial microtubules, as well as reduced astral polar microtubules (Fig. 5A). Analysis of β-tubulin fluorescence intensity at the equatorial region between anaphase and telophase revealed a 1.6-fold increased labelling when hepatocytes completed cytokinesis, whereas no significant increase was observed when cells did not complete (supplementary material Fig. S3).
Incomplete cytokinesis and liver growth

Because astral microtubules become stabilized upon contacting the cortex (Burgess and Chang, 2005), we next analyzed the interaction between astral microtubules and the equatorial cortex. We demonstrated that during incomplete cytokinesis, astral microtubules failed to contact the equatorial cortex (Fig. 5B). Furthermore, we analyzed localization of EB1, a protein that only associates with elongating microtubules and not with microtubules that become stabilized upon contact with the cortex (Strickland et al., 2005). We observed that the equatorial cortical EB1 staining was weak in hepatocytes that completed cytokinesis (Fig. 5C), reflecting the association of the majority of astral equatorial microtubules to the cortex. By contrast, when cells did not complete cytokinesis, EB1 was still present on microtubule tips, illustrating the absence of anchorage (Fig. 5C). Taken together, these results demonstrate that, during incomplete cytokinesis, the absence of astral microtubules anchorage to the equatorial cortex induces a total destabilization of the microtubule network.

The equatorial zone of active RhoA is absent during the incomplete cytokinesis process

We next hypothesized that the signal delivered by microtubules to the cortex must be impaired in this specific liver-division programme. Recent studies clearly demonstrate that proteins that localize to astral and/or central spindle microtubules are delivered to the equatorial cortex in order to activate the Rho GTPase RhoA, which is indispensable to induce furrowing (Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yoshizaki et al., 2004; Yuce et al., 2005). In this context, we analyzed the localization of MgcRacGAP (centralspindlin protein), Aurora B (chromosomal passenger protein) and PRC1 (maintenance of central spindle) (Eggert et al., 2006). If hepatocytes completed cytokinesis, MgcRacGAP was present during anaphase both on the central spindle and on astral equatorial microtubules and during telophase at the midbody (Fig. 6A). During incomplete cytokinesis, MgcRacGAP was localized on the remaining interdigitating microtubules in anaphase B and telophase but was never observed on unattached astral equatorial microtubules (Fig. 6A). The same profile was observed for other microtubule-associated proteins, such as aurora B, PRC1 and Plk1 (supplementary material Fig. S4). These results argue the fact that, during the incomplete cytokinesis process, microtubule-dependent molecular signal is not delivered to the equatorial cortex. Finally, we investigated RhoA localization. As described before for other cell types (Yoshizaki et al., 2003; Yoshizaki et al., 2004), RhoA accumulated at the equatorial cortex in early telophase in hepatocytes that complete cytokinesis; then it concentrated at the cleavage furrow and finally at the midbody (Fig. 6B). During incomplete cytokinesis, RhoA did not localize to the cortex.
correctly localize at the equatorial cortex, in which astral microtubules were almost lost; we observed a diffuse repartition near the cell centre close to central spindle microtubules (Fig. 6B). This result is in agreement with other studies that have demonstrated that RhoA zones are diffuse when microtubules are distant from the cortex (Bement et al., 2005; Nishimura and Yonemura, 2006). In conclusion, our results indicate that, during incomplete cytokinesis, molecular signals delivered by microtubules to the equatorial cortex are impaired, preventing the activation of RhoA pathway.

Discussion
Incomplete cytokinesis is increasingly recognized as an important source of genomic instability taking place during cell transformation (Fujiwara et al., 2005; Shi and King, 2005; Storchova and Pellman, 2004). Paradoxically, incomplete cytokinesis is also associated with developmental programmes in different species (Glotzer, 2001). In this study, we identified, in the liver, a new developmental process of incomplete cytokinesis triggered by weaning. Observation of hepatocytes during the binucleation process demonstrates that, although karyokinesis is normally accomplished, the cleavage plane is never specified in these cells. Cardiomyocytes are the most related physiological model that, like hepatocytes, perform karyokinesis but only partially assemble the actomyosin ring (Engel et al., 2006). Nevertheless, in our system, binucleation was never associated with mitotic abnormalities that lead to nuclear bridging and micronuclei; these tetraploid progenies will be able to execute a new cell division cycle (Guidotti et al., 2003). It is now clear that the onset of animal cell cytokinesis must be precisely controlled in both time and space in order to promote correct furrow formation (D’Avino et al., 2005; Wang, 2005). Assembly and dynamic turnover of actin and myosin II at the equatorial cortex is required to induce cell shape modification. By following the divisions of living cells after weaning, we established that anaphase cell elongation is clearly impaired during incomplete cytokinesis. This phenotype has been confirmed in vivo; telophase hepatocytes presenting no membrane ingression kept the same shape as in metaphase. Moreover, we demonstrate that the actin cytoskeleton is not reorganized to the cleavage plane during anaphase-telophase transition. In Drosophila cells, this process has been shown to be controlled by the ROCK/myosin II pathway (Dean and Spudich, 2006; Hickson et al., 2006). Myosin II is recruited to the equatorial cortex of the cell by a Rho kinase-dependent mechanism, where it will contribute to cell elongation through a broad equatorial elongation prior to the more-restricted contraction of the actomyosin ring. We demonstrate that this pathway is conserved in mammalian cells. In our model, ROCK activity is clearly impaired after weaning; phospho-RLC is not recruited to the equatorial cortex leading to the absence of actin cytoskeleton reorganization. From studies in many laboratories, it is clear that communications between microtubules and the actin cortex somehow direct the activation of RhoA in a precisely defined zone at the equator, promoting rapid remodelling of the cortical actomyosin cytoskeleton (Kamijo et al., 2006; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Yoshizaki et al., 2004; Yuce et al., 2005). In particular, astral microtubules emanating from the spindle poles and central spindle microtubules between separating chromosomes are essential for the activation of equatorial cortical regions (D’Avino et al., 2005). Indeed numerous signalling proteins – including the centralspindlin complex MKLP1 and MgcRacGAP, the passenger protein Aurora B, and the Rho GTPase exchange factor ECT2 – are involved in relaying information from the spindle to the cortex and ultimately anchor the crucial cytokinesis activator RhoA (Eggert et al., 2006). We show that, in hepatocytes that do not complete cytokinesis, microtubule network behaviour is normal until early anaphase; thereafter, elongating microtubules fail to interact with the equatorial cortex. As a consequence, the spindle collapses in telophase. Consistent with these results, we demonstrate that the microtubule molecular signal essential for furrow induction is not delivered to the equatorial cortex. Consequently, active RhoA does not concentrate at the putative site of furrow formation, leading to an absence of activation of its downstream signals. Finally, it is quiet captivating that a specific cell division programme can be controlled by animals weaning. In the liver, weaning is the trigger of complex physiological changes, such as nutriment/hormonal balance and modification of the circadian cycle (Gupta, 2000). Further studies will be needed to show whether one or several of these factors could control astral equatorial cortical capture by modifying either the integrity of plus-tips proteins, which are essential to mediate cortical interactions, and/or cell membrane composition, preventing anchorage of astral equatorial microtubules to the cortex.

Materials and Methods

Animals
Male Wistar rats (IFFACREDO, France) were treated in accordance with European Union regulations on animal care. The rats were housed under standard light/dark conditions and received pelleted food and water ad libitum. All rats were weaned at 19 days after birth.

Cell cultures and inhibitor
Hepatocytes were isolated from rat livers by a two-step perfusion as previously described (Guidotti et al., 2003). Latrunculin A (Calbiochem) was used at 2 μM; HA1077 (Sigma-Aldrich) was used at 10 μM.

Antibodies
Commercial primary antibodies used were as follows: mouse anti-β-catenin (BD Transduction Laboratories, 1:200), mouse anti-β-tubulin (Tub 21, Sigma, 1:400), mouse anti-RhoA (Santa Cruz, 1:100), mouse anti-E1B (BD Transduction Laboratories, 1:100), mouse anti-GS (BD Transduction Laboratories, clone 6, 1:200), mouse anti-phospho-Mycin light chain 2 (nonmuscle) (3675, Cell Signaling, 1:50), mouse anti-AIM-1 (BD Transduction Laboratories, 1:100), mouse anti-Plk1 (Euromedx-upstate, clone 35-206, 1:200), rabbit anti-Pepck1 (from Lamers, AMC Liver Center, Amsterdam, The Netherlands, 1:1000), rabbit anti-MgcRacGAP (from T. Kitamura, Institute of Medical Research Science, Tokyo, Japan, 1:1000), rabbit anti-PRC1 (from W. Jiang, Burnham Institute for Medical Research, La Jolla, CA, 1:100) and 165 nM Alexa-Fluor-488-phallolidin (Molecular probes). Secondary antibodies to rabbit and mouse IgG were conjugated either with Alexa-Fluor-488 or Alexa-Fluor-594 (Molecular probes, 1:500).

Live imaging
Hepatocytes were grown on 35 mm coverslips coated with collagen solution (Sigma) and mounted on the microscope after 60 hours of culture. During imaging, hepatocytes were on a stage heated at 37°C under a 5% CO2 atmosphere. Cells were filmed every 90 seconds with a Leica DMRBE using a 63X lens (numerical aperture, 1.4), a condenser (working distance, 23 mm; numerical aperture, 0.53) and a Pentamorph cooled CCD camera (Popper Scientific) coupled to an electronic shutter. Metamorph 7.1 was used for computer-based image acquisition and analysis of live cell data. The single images shown were prepared using Adobe Photoshop CS.

Immunohistochemistry
Liver tissues fixed in 10% phosphate buffered formalin were embedded in paraffin. Tissue sections (3 μm) were obtained using a conventional microtome. Sections
were deparaffinized in xylene and placed in 100% ethanol. Sections were rehydrated in a descending gradient of ethanol-water and then boiled for 2-5 minutes in Tris 0.1 M Tween 0.1%. All subsequent antibody incubations were carried out as described below.

Immunofluorescence

Coverslips containing hepatocytes were collected and washed in PBS. The cells were fixed either in -20°C MeOH for 5 minutes (to stain β-tubulin, β-catemin, EB1, MgcRacGAP, Aurora B, Pr and Plk1), or in 4% TCA for 15 minutes (to stain RhoA), or in 4°C 4% PFA for 15 minutes (to stain actin and phospho-RLC). After blocking in PBS with 10% goat serum (30 minutes), cells were incubated for 1 hour with primary antibodies, washed in PBS containing 0.1% Tween 20 and incubated with secondary antibodies for 30 minutes, all at room temperature. Hoechst 33342 (0.2 μg/ml, Sigma) was included in the final wash to counterstain nuclei. Samples were mounted on slides in Fluorescent mounting medium.

Image acquisition and analysis

Images were taken using a Nikon Statik E360 microscope with 60× magnification, 1.4-0.7 NA PL-APO objectives, a DXM1200 cooled CCD camera (Universal Imaging). A total of 20-30 planes (0.2μm) of all cell and compiled as single two-dimensional projections using ImageJ software. All images were imported into Adobe Photoshop CS for contrast manipulation and image acquisition and analysis.

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