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Loss of Survivin influences liver regeneration and is associated with impaired Aurora B function

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The chromosomal passenger complex (CPC) acts as a key regulator of mitosis, preventing asymmetric segregation of chromosomal material into daughter cells. The CPC is composed of three non-enzymatic components termed Survivin, the inner centromere protein (INCENP) and Borealin, and an enzymatic component, Aurora B kinase. Survivin is necessary for the appropriate separation of sister chromatids during mitosis and is involved in liver regeneration, but its role in regenerative processes is incompletely elucidated. Whether Survivin, which is classified as an inhibitor of apoptosis protein (IAP) based on domain composition, also has a role in apoptosis is controversial. The present study examined the *in vivo* effects of Survivin ablation in the liver and during liver regeneration after 70% hepatectomy in a hepatocyte-specific knockout mouse model. The absence of Survivin caused a reduction in the number of hepatocytes in the liver, together with an increase in cell volume, macronucleation and polyploidy, but no changes in apoptosis. During liver regeneration, mitosis of hepatocytes was associated with mislocalization of the members of the CPC, which were no longer detectable at the centromere despite an unchanged protein amount. Furthermore, the loss of survivin in regenerating hepatocytes was associated with reduced levels of phosphorylated Histone H3 at serine 28 and abolished phosphorylation of CENP-A and Hec1 at serine 55, which is a consequence of decreased Aurora B kinase activity. These data indicate that Survivin expression determines hepatocyte number during liver development and liver regeneration. Lack of Survivin causes mislocalization of the CPC members in combination with reduced Aurora B activity, leading to impaired phosphorylation of its centromeric target proteins and inappropriate cytokinesis.
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Survivin, the smallest member of the family of apoptosis proteins, is exhibiting unique properties that interfere with apoptosis and promote cell cycle progression.[1,2] Survivin is highly expressed in various malignancies, but is also detected in several differentiated adult tissues, especially during proliferation and regenerative processes.[3–7] Recently, it has also been shown that Survivin has several mitotic functions and has a central role in cell division.[2,8] As a member of the chromosomal passenger complex (CPC), Survivin has a crucial role in chromosome segregation during mitosis and cytokinesis. The CPC acts as a key integrator of chromosomal and cytoskeletal events to assure proper completion of mitosis and prevent unequal segregation of chromosomal material into daughter cells. CPC localization during the cell cycle is linked to its mitotic functions.[9] The CPC consists of three non-enzymatic components, Survivin/Bir1, the inner centromere protein (INCENP) and Borealin/DasraB, all of which regulate the activity, localization, stability and likely the substrate specificity of its enzymatic component, Aurora B kinase.[10] The substrates of Aurora B kinase include Ser10 and Ser28 of Histone H3, the human centromeric protein A (CENP-A), a centromeric variant of Histone H3, and the microtubule motor protein Mklp1.[11–13] Survivin binds to the N-terminal end of INCENP, stabilizing the CPC and promoting its centromere targeting.[10,14] Recent studies imply that targeting of the CPC to the inner centromere and activation of Aurora B kinase depends on the phosphorylation of Histone H3 at threonine 3 by Haspin kinase, which is subsequently recognized by Survivin.[15–17] While a global knockout of Survivin in mice is embryonically lethal,[18] several conditional or tissue-specific knockout models have been described. Mice with a cardiomyocyte-specific deletion of Survivin developed progressive heart failure due to a decreased total number of cardiomyocytes.[19] In studies conducted in rodents, an

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Abbreviations: CPC, chromosomal passenger complex; INCENP, inner centromere protein; IAP, inhibitor of apoptosis protein; CENP-A, centromeric protein A; Alb-surv$^{-/-}$, albumin-cre survivin$^{flox/flox}$; pHistone H3(S10), phosphorylated Histone H3 at serine 10; pHistone H3(S28), phosphorylated Histone H3 at serine 28; pHec-1(S55), phosphorylated highly expressed in cancer protein 1 at serine 55; pCENP-A(S7), phosphorylated centromeric protein A at serine 7; VRK-1, vaccinia-related kinase 1; ALAT, serum alanine-aminotransferase; ASAT, serum aspartate-aminotransferase

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increase in Survivin expression after partial hepatectomy was observed at postoperative days 2–3.[3,4] After split liver transplantations in humans, Survivin expression was elevated in the graft at day 5. In both settings, Survivin immunoexpression correlated with proliferation but not with apoptosis.

In summary, these data suggest that Survivin is expressed in the liver and shows an association with proliferating cells. Several studies performed *in vitro* examined the role of Survivin in mitosis. The heart-specific conditional knockout model supported the notion that *in vivo*, Survivin functions in cell division during organ development. However, further investigations were limited because mitosis is virtually absent in the adult rodent heart. To our knowledge, the effects of Survivin deletion in non-malignant tissue proliferation are not well understood. In this study, we therefore generated a conditional hepatocyte-specific Survivin knockout mouse model, allowing us to evaluate the influence of Survivin on mitosis *in vivo* by liver regeneration after partial hepatectomy using immunohistochemistry, morphometric analyses, western blotting and kinase assays. This study for the first time examines the *in vivo* consequences of missing Survivin on mitosis, proliferation and apoptosis in organ regeneration at the cellular and molecular level.

Results

Alb-surv$^{-/-}$ mice exhibit no differences in phenotype compared with wild-type littermates under non-stress conditions. To generate mice lacking hepatic Survivin, mice with floxed Survivin alleles[7] were crossed with mice transgenic for Cre-recombinase under the control of the albumin promoter. Hepatocyte-specific deletion of Survivin at a gene level was confirmed by laser capture microdissection (Figure 1a) and subsequent single-cell PCR (Figure 1b). Comparison of the liver–bodyweight ratio of wild-type and Alb-surv$^{-/-}$ mice revealed no differences (Figure 1c).

Increased cell volume and nuclear-DNA content with reduced total hepatocyte numbers in Survivin-deficient livers. Macroscopic examination of the livers from wild-type and Alb-surv$^{-/-}$ mice did not reveal any differences. Hematoxylin and eosin (H&E) staining of liver sections indicated that general architecture was retained in both the genotypes (Figure 2a). Portal tracts were normal and showed equal amounts of fibrous tissue (data not shown). However, hepatocytes of Alb-surv$^{-/-}$ mice were massively enlarged with big nuclei displaying a coarsely granulated chromatin pattern and frequent vacuoles. Based on these findings and the fact that there were no genotype-dependent differences in liver–body weight ratios, we determined the total number, volume and DNA content of the hepatocytes. Compared with wild-type hepatocytes, Alb-surv$^{-/-}$ hepatocytes were markedly decreased in number (Figure 2b), but increased in volume (Figure 2c), and nuclei had increased DNA content (Figure 2d).

The role of Survivin in liver regeneration. We sought to elucidate the cause of decreased total number of hepatocytes and the consequences of depleting Survivin in proliferating hepatocytes. To that end, a liver regeneration model was used in which 70% of the liver was surgically

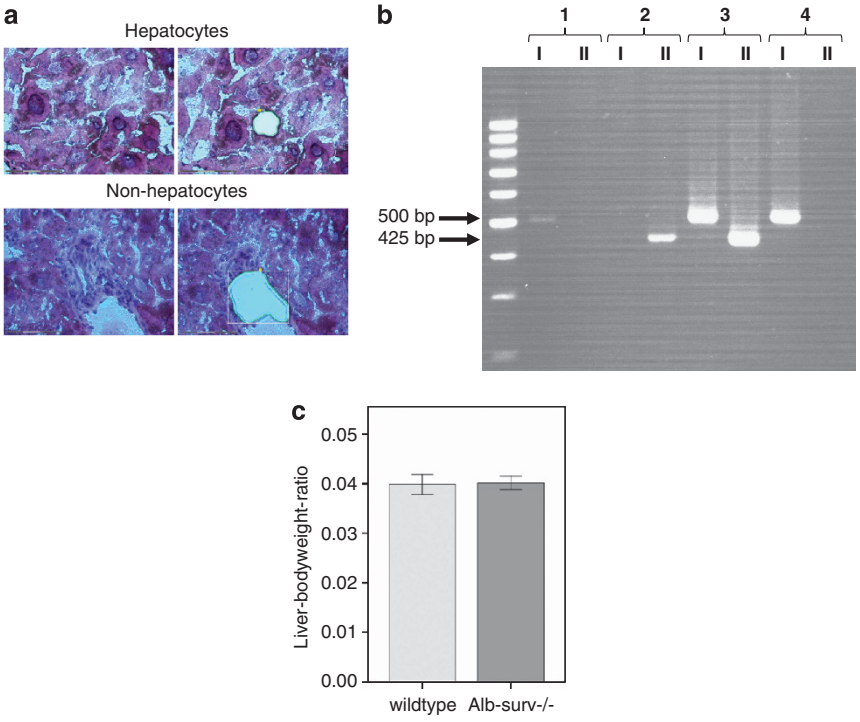

Figure 1 Determination of the hepatocyte-specific Survivin knockout and the effect on liver–body weight ratio. (**a**) Representative image of microdissection of hepatocytes and non-hepatocytes for DNA isolation. (**b**) Survivin-specific (I) and Survivin deletion-specific (II) PCR results of microdissected non-hepatocytes (1) and hepatocytes (2), DNA preparation complete liver section (3) and DNA preparation complete heart section (4). Survivin-specific and Survivin deletion-specific PCR results in a band at 500 bp and 425 bp, respectively. (**c**) Liver–body weight ratio of wild-type and liver-specific Survivin-knockout mice (Alb-surv$^{-/-}$) at 8–12 weeks of age (mean ± S.E.)

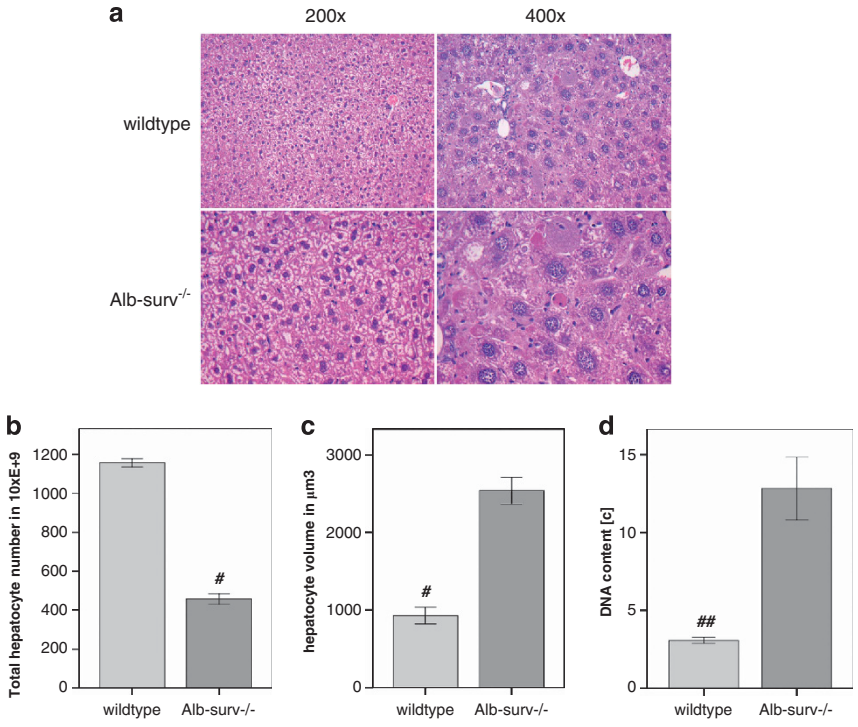

Figure 2 Histologic analysis of liver-specific Survivin-knockout livers (Alb-surv$^{-/-}$). (**a**) H&E staining of liver sections from wild-type- and Alb-surv$^{-/-}$ mice visualized at $\times$ 200 (left) and $\times$ 400(right) magnification. Analysis of (**b**) total hepatocyte number, (**c**) hepatocyte volume and (**d**) DNA content per cell of wild-type and Alb-surv$^{-/-}$ mice livers at 8–12 weeks of age (mean ± S.E.). $^{\#}P<0.05$; $^{\#\#}P<0.01$

removed and the responses were monitored for the subsequent 14 days.

To calculate the liver–body weight ratio, only the liver weight at the point of sacrifice was used (Figure 3a). At day 3 post surgery, a small difference between the genotypes was noted. Otherwise, there was a general increase in the ratio as a sign of liver regeneration, and no genotype-dependent differences were noted. In Alb-surv$^{-/-}$ mice, serum levels of ALAT were higher after 24 h of regeneration compared with that in wild-type mice (Figure 3b). For serum levels of ASAT, no differences between the two genotypes could be observed throughout the duration of the experiment (Figure 3c). Histologically, these livers revealed spotty necrosis of hepatocytes (data not shown). During liver regeneration, the wild-type mice responded with an increase in total hepatocyte number at days 2, 7 and 14. However, such an increase was absent in Alb-surv$^{-/-}$ mice (Figure 3d), whereas hepatocyte volume was constantly higher compared with that in the wild-type (Figure 3e).

Reduced number of total hepatocytes during regeneration of Survivin-deficient livers is associated with decreased proliferative activity. Hepatocyte proliferation was analyzed using Ki-67 immunohistochemical staining. Increased Ki-67 was detectable at day 2 after partial hepatectomy in wild-type and Alb-surv$^{-/-}$ mice (Figure 4a). The highest levels of Ki-67-positive cells were observed at day 2 for wild-type livers and day 3 for Survivin-deficient livers, respectively, and a constant decline in proliferation was observed until day 14 post surgery.

Counting of mitotic figures in sections of regenerating livers showed a peak at day 3 after partial hepatectomy for both genotypes (Figure 4b). In both settings, Alb-surv$^{-/-}$ mice showed considerably lower values compared with wild-type mice. There was no evidence of increased apoptosis of hepatocytes based on the Caspa-Tag *in-situ* assay (Figure 4c) or TUNEL assay (not shown) at any time during post-surgery liver regeneration. In agreement with the initial finding, DNA content was markedly elevated in Alb-surv$^{-/-}$ hepatocytes (Figure 4d). Moreover in Alb-surv$^{-/-}$ hepatocytes, DNA content continually increased until day 14, while wild-type hepatocytes had fairly constant DNA levels throughout regeneration.

Loss of Survivin leads to disturbed localization of the chromosomal passenger complex. Survivin, Aurora B and INCENP are members of the CPC that is responsible for histone (H3) phosphorylation and chromosome segregation during mitosis. The observed phenotype of decreased number but increased volume and DNA content of hepatocytes could be due to disturbed mitosis in Survivin-deficient livers. In particular, this may be due to a failure in completing cytokinesis, a function in which Survivin has previously been implicated. Consequently, these proteins were analyzed 3 days post surgery by immunofluorescent colocalization studies. This time point was selected, as mitosis was determined to be maximal in both genotypes.

In wild-type pro- and metaphase hepatocytes, there was a clear punctuated colocalization of Survivin and Aurora B at the chromosomes (Figure 5a). As expected, no signal for Survivin

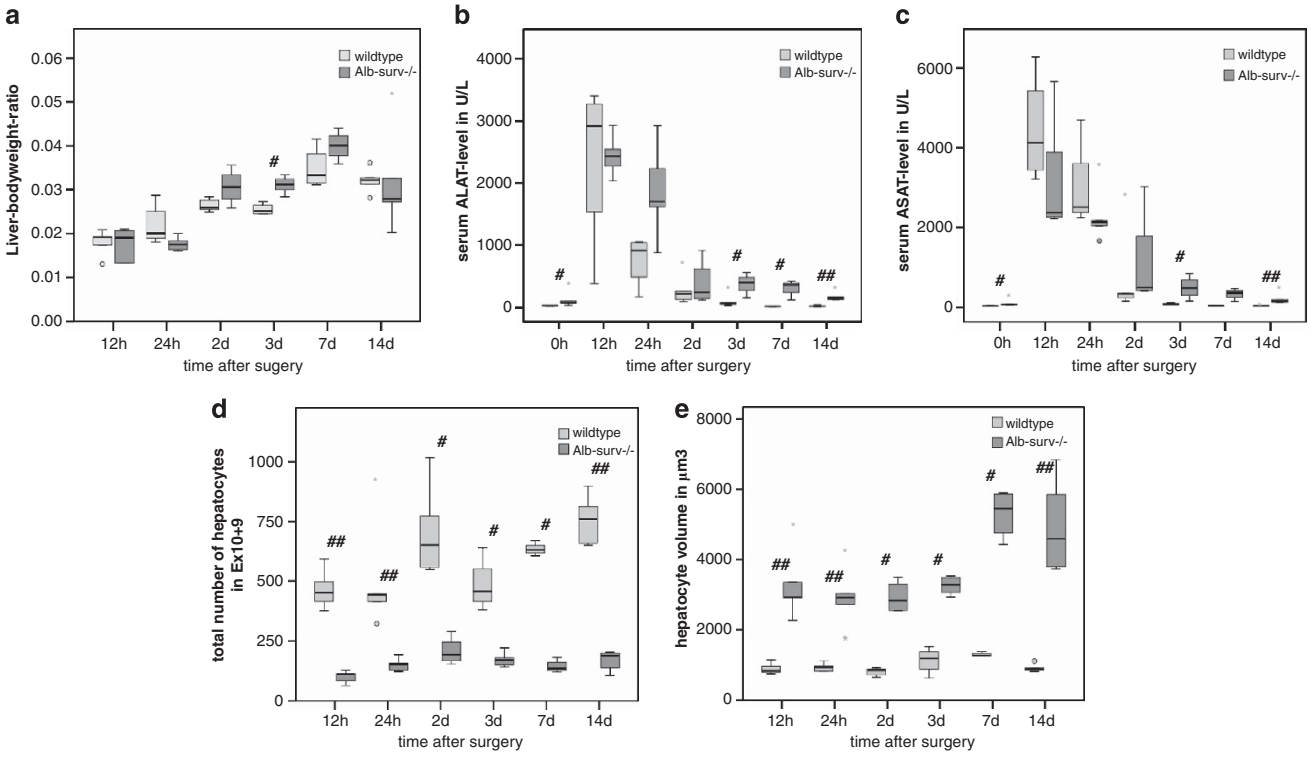

Figure 3 Genotype-specific differences in liver regeneration experiments. Wild-type and liver-specific Survivin knockout (Alb-surv$^{-/-}$) mice underwent 70% hepatectomy for liver regeneration experiments. Mice were sacrificed after 12 and 24 h, and 2, 3, 7 and 14 days. Subsequently (**a**) liver–body weight ratio, (**b**) serum alanine-aminotransferase (ALAT) levels and (**c**) serum aspartate-aminotransferase (ASAT) levels were determined. (**d**) Total hepatocyte number and (**e**) hepatocyte volume were analyzed in wild-type and Alb-surv$^{-/-}$ livers at each time point. $^{\#}P<0.05$; $^{\#\#}P<0.01$

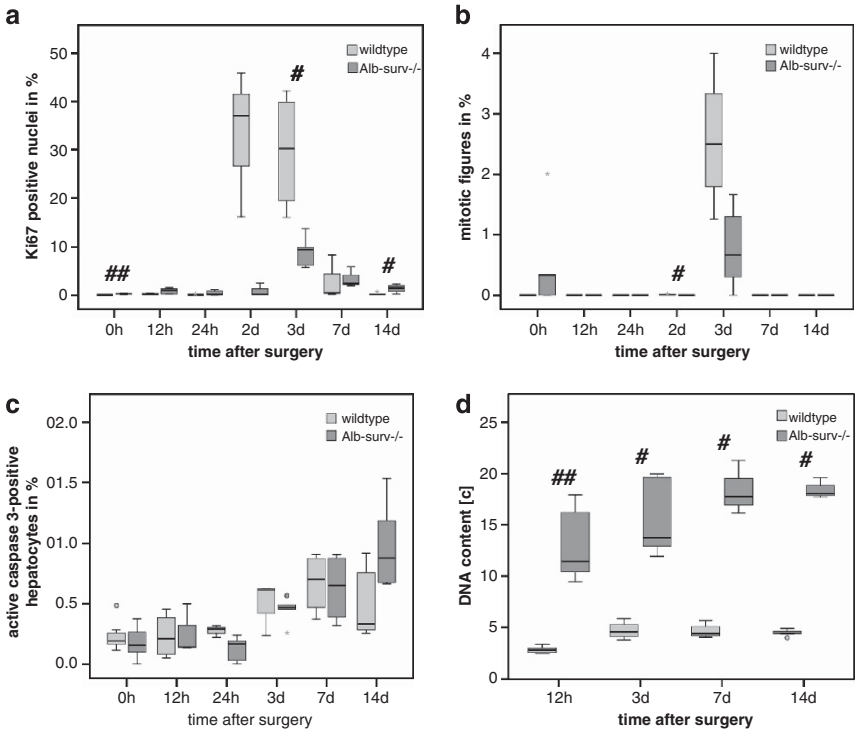

Figure 4 Analysis of proliferation and apoptosis after 70% hepatectomy. Wild-type and liver-specific Survivin (Alb-surv$^{-/-}$) mice underwent 70% hepatectomy and were sacrificed after 12 and 24 h, and 2, 3, 7 and 14 days after surgery. Liver sections were analyzed for (**a**) the proliferation marker Ki-67, (**b**) mitotic figures, (**c**) Active caspase 3-positivity and (**d**) DNA contents. $^{\#}P<0.05$; $^{\#\#}P<0.01$

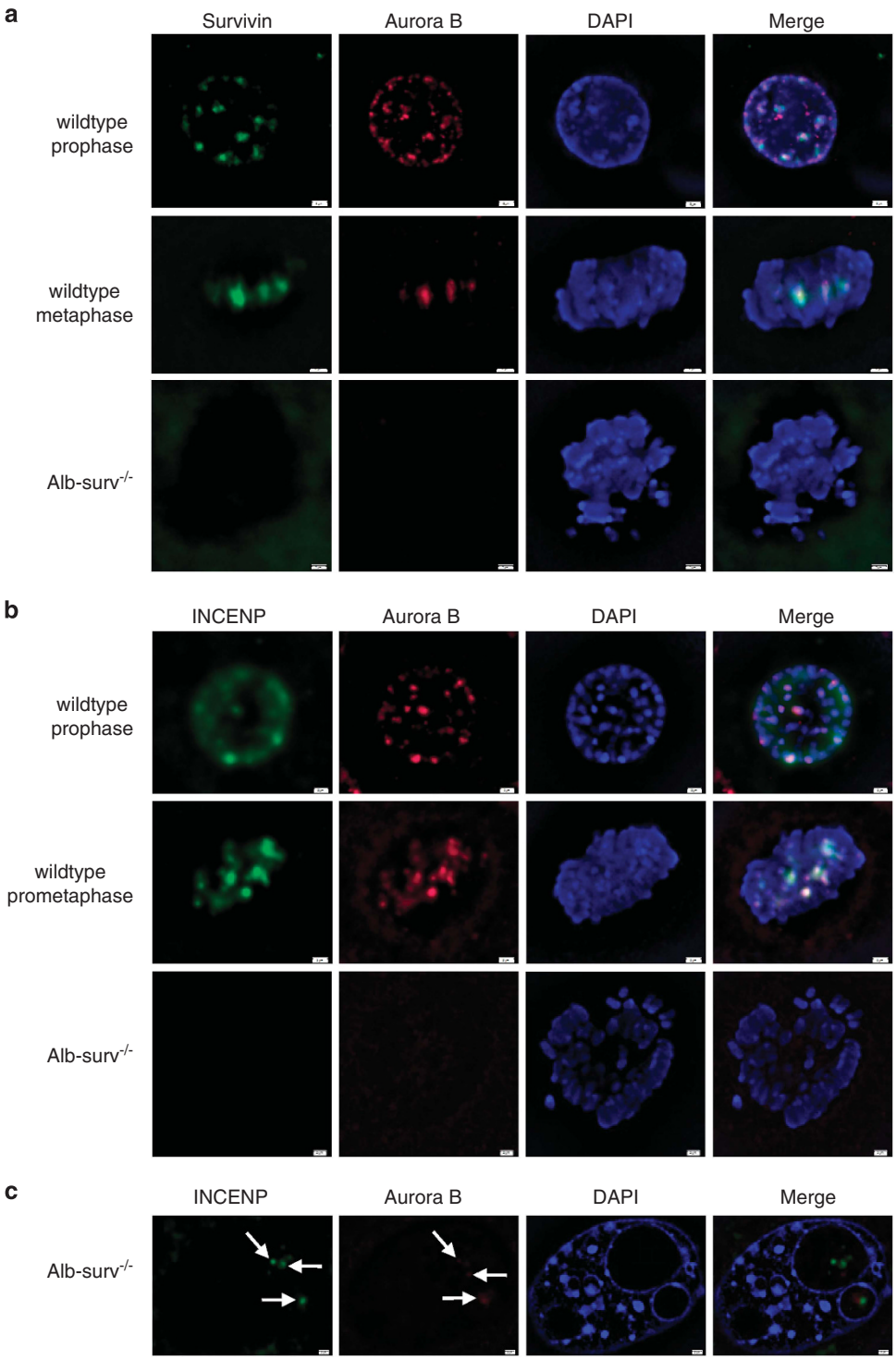

Figure 5 Effects of Survivin deletion 3 days after 70% liver resection. Immunofluorescent double labeling of Survivin and INCENP with Aurora B in mitosis of wild-type and Alb-surv$^{-/-}$ hepatocytes. (**a**) Wild-type prophase and metaphase show Aurora B (red) colocalized with Survivin (green). In Alb-surv$^{-/-}$ mitosis, Aurora B and Survivin are not detectable. (**b**) Immunofluorescence images of colocalized Aurora B (red) and INCENP (green) in wild-type prophase and prometaphase. Alb-surv$^{-/-}$ mitosis shows a complete lack of Aurora B and INCENP. (**c**) In Alb-surv$^{-/-}$ hepatocyte interphase nucleus, Aurora B (red, arrows) and INCENP (green, arrows) are detectable in vacuoles without colocalization. All sections were additionally stained with DAPI. White bars indicate 2 μm. (**d**) Protein expression levels of the CPC proteins Survivin, Aurora B, INCENP and Histone H3 phosphorylated at serine 10 (S10) and serine 28 (S28) determined in liver lysates by western blotting. Expression levels were determined by densitometry and normalized to GAPDH. For phosphorylated Histone H3(S10) and Histone H3(S28), an additional normalization to the number of mitotic events and averaged DNA content was performed. Wild-type expression was set to 100%. Significant reduction of Survivin protein expression in Alb-surv$^{-/-}$ liver lysates was detected, whereas the reduced phosphorylation of Histone H3(S28) did not reach significance. $^{\#\#}P<0.01$. (**e**) Aurora B-activity was determined by *in-vitro* kinase assays at 0 h and day 3 post surgery. In Alb-surv$^{-/-}$ lysates the Aurora B kinase activity was significantly reduced 3 days post surgery, compared with wild type. As a control, wild-type lysates day 3 post surgery were treated with the Aurora B-inhibitor ZM447439. Activity of wild-type lysates at 0 h were set to 100%. All data are mean ± S.E. $^{*}P<0.05$ *versus* all other conditions; $^{\#}P<0.05$ *versus* day 3 post-surgery wild-type and Alb-surv$^{-/-}$

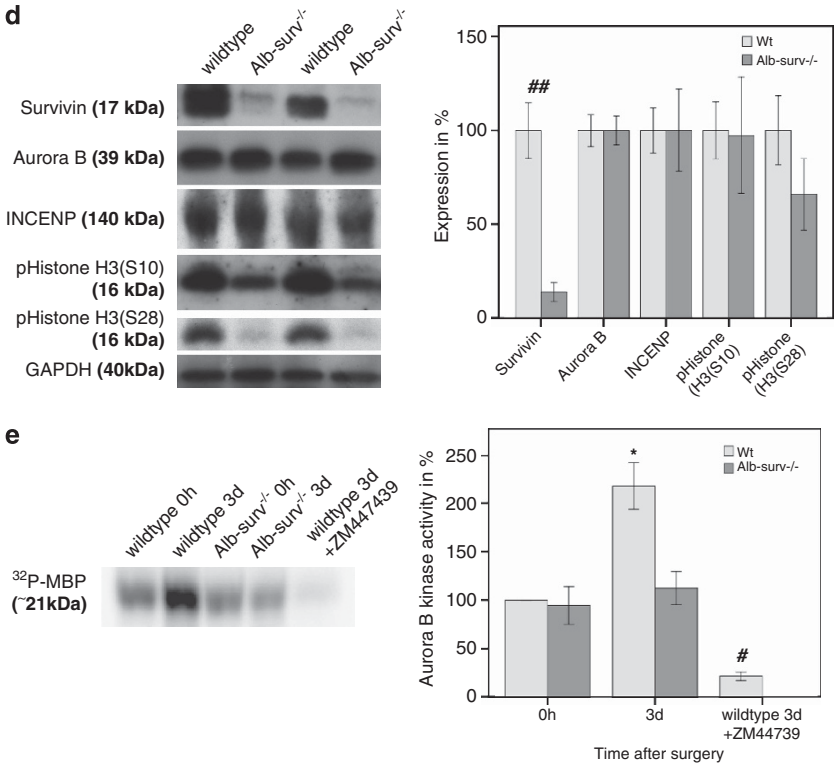

Figure 5 (Continued)

was detectable in mitotic Alb-surv$^{-/-}$ hepatocytes. Interestingly, along with the loss of Survivin, the signals for Aurora B were also nearly completely undetectable. Similar findings were observed for localization of INCENP and Aurora B (Figure 5b). In addition, several Alb-surv$^{-/-}$ hepatocytes, at the interphase, exhibited Aurora B and INCENP immunoreactivity in nuclear vacuoles (Figure 5c). Aurora B was also detectable in the cytoplasm of some Alb-surv$^{-/-}$ hepatocytes undergoing mitosis without an association with chromosomal DNA (data not shown).

Phosphorylation of Histone H3(S28) is reduced in regenerating Survivin-deficient livers without changes in phosphorylated Histone H3(S10). At day 3 after surgery, there was high mitotic activity in regenerating livers of both genotypes with mislocalization of the CPC proteins in Survivin-deficient livers. We analyzed whether this resulted in a change in expression levels of the phosphorylation state of Histone H3(S10) and Histone H3(S28), both substrates of the CPC. Western blot analyses of total liver protein extracts 3 days after 70% hepatectomy (Figure 5d) revealed that Survivin protein expression was decreased to 19% of the wild-type levels. Protein levels of Aurora B and INCENP did not change when compared with the wild-type. However, interestingly, a clear decrease in phosphorylation of Histone H3(S28) was detected whereas no change for phosphorylated Histone H3(S10) was observed. Regenerating livers of Alb-surv$^{-/-}$ mice showed only 66% of phosphorylated Histone H3(S28) levels normalized to the wild-type, the number of mitotic events and DNA content.

Survivin deficiency leads to reduced Aurora B activity in regenerating livers. To determine whether decreased Histone H3(S28) phosphorylation might be due to reduced Aurora B activity, kinase assays were performed for wild-type and Survivin-deficient total liver extracts at the start of ($t=0$) and after 3 days of regeneration induction (Figure 5e). Wild-type livers showed an increase in Aurora B-activity up to 218% at day 3 after 70% hepatectomy compared with the level before surgery, whereas nearly no induction was seen in Alb-surv$^{-/-}$ livers (92% Aurora B-activity before and 112% at day 3 after 70% hepatectomy, normalized to wild-type ($t=0$) pre-surgery situation). As controls, lysates of wild-type livers 3 days post surgery were treated with the Aurora B-inhibitor ZM447439, which almost completely abolished kinase activity.

Aurora B substrates Histone H3(S28), CENP-A and Hec1 are no longer phosphorylated during mitosis in Alb-surv$^{-/-}$ hepatocytes. Due to the results of the colocalization studies and the reduced Histone H3(S28) phosphorylation, combined with nearly no induction of Aurora B in Alb-surv$^{-/-}$ hepatocytes during regeneration, we analyzed whether other substrates of Aurora B were also affected by altered phosphorylation. Therefore, we performed immunofluorescent colocalization studies of Aurora B with pHistone H3(S10), pHistone H3(S28), pCENP-A and pHec1(S55) (Figure 6).

In wild-type hepatocyte mitosis, pHistone H3(S10) and (S28) showed colocalization with Aurora B and chromosomal DNA (Figures 6a and b). In Alb-surv$^{-/-}$ hepatocyte mitosis, phosphorylation of pHistone H3(S10) was preserved,

however, no signal for pHistone H3(S28) at chromosomal DNA was detected. Phosphorylation of CENP-A(S7) and Hec1(S55) was also not detectable (Figures 6c and d).

Discussion

Loss of Survivin in animal cells or its homolog in evolutionarily less developed organisms results in severe defects in proliferation processes, including polyploidy, macro- and multinucleation, and an increase in apoptosis.[18,20,21] Mice specifically lacking hepatic Survivin did not exhibit any macroscopic alterations compared with wild-type littermates. Although no changes in general liver architecture were detectable, Survivin-deficient livers notably exhibited reduced hepatocyte cell numbers combined with a marked increase in hepatocyte volume and increased DNA content of the nuclei.

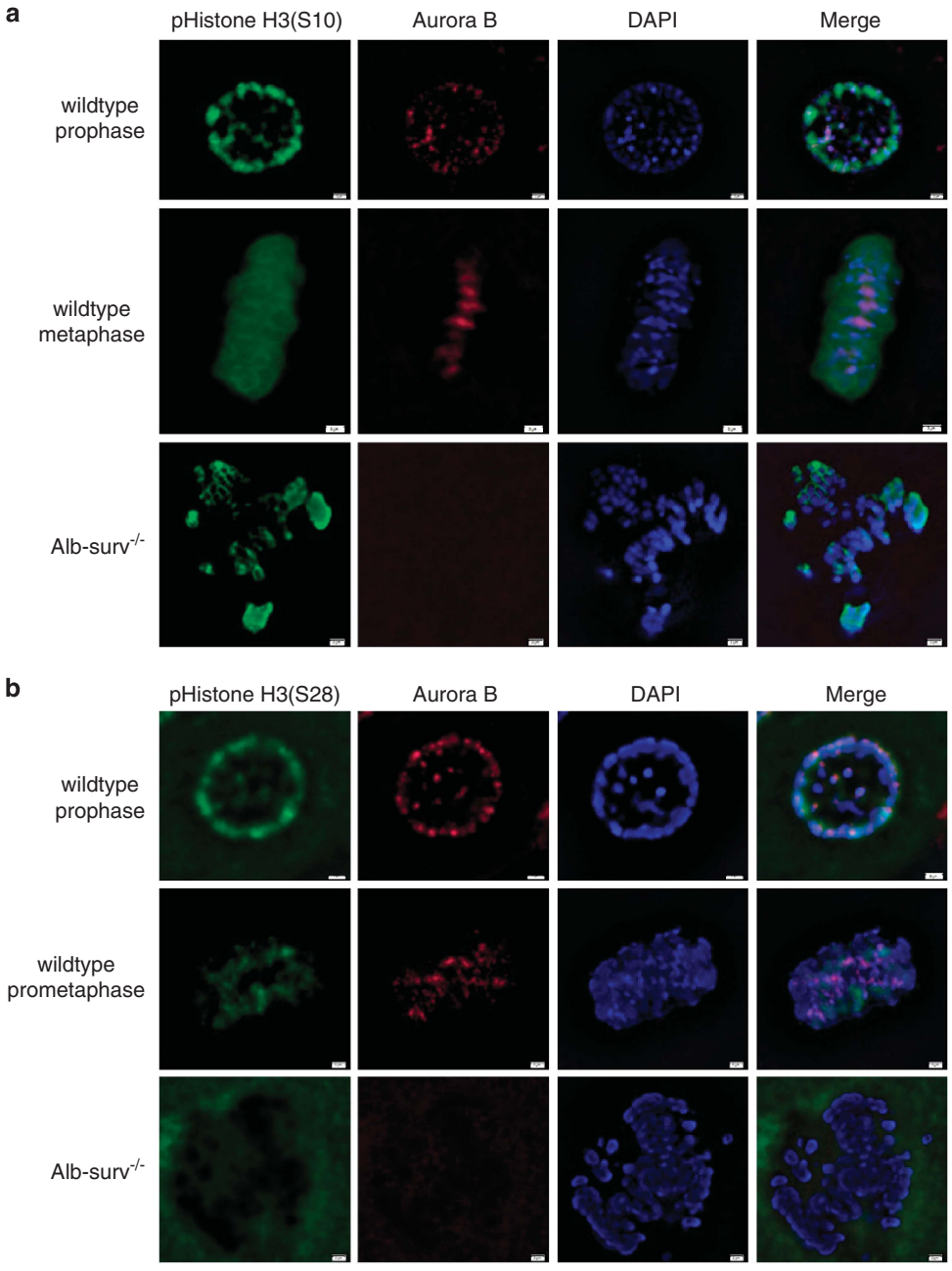

Figure 6 Immunofluorescent double labeling of Aurora B with its centromeric substrates after 3 days of regeneration. (**a**) Wild-type prophase and metaphase show Aurora B (red) and pHistone H3(S10) (green). However, in Alb-surv$^{-/-}$ mitosis, the pHistone H3(S10) (green) is preserved despite of the lack of Aurora B. (**b**) Wild-type prophase and prometaphase show colocalization of Aurora B (red) and pHistone H3(S28) (green) whereas Alb-surv$^{-/-}$ mitosis is completely negative for Aurora B and pHistone H3(S28). (**c**) Colocalization of pCENP-A(S7) (green) with Aurora B (red) in wild-type prophase and prometaphase. In Alb-surv$^{-/-}$ mitosis, no signals are detectable for pCENP-A and Aurora B. (**d**) Colocalization of pHec 1(S55) (green) with Aurora B (red) in wild-type prophase and metaphase; however, in Alb-surv$^{-/-}$ mitosis, no immunoreactivity is shown for both proteins. All sections were additionally stained with DAPI. White bars indicate 2 μm

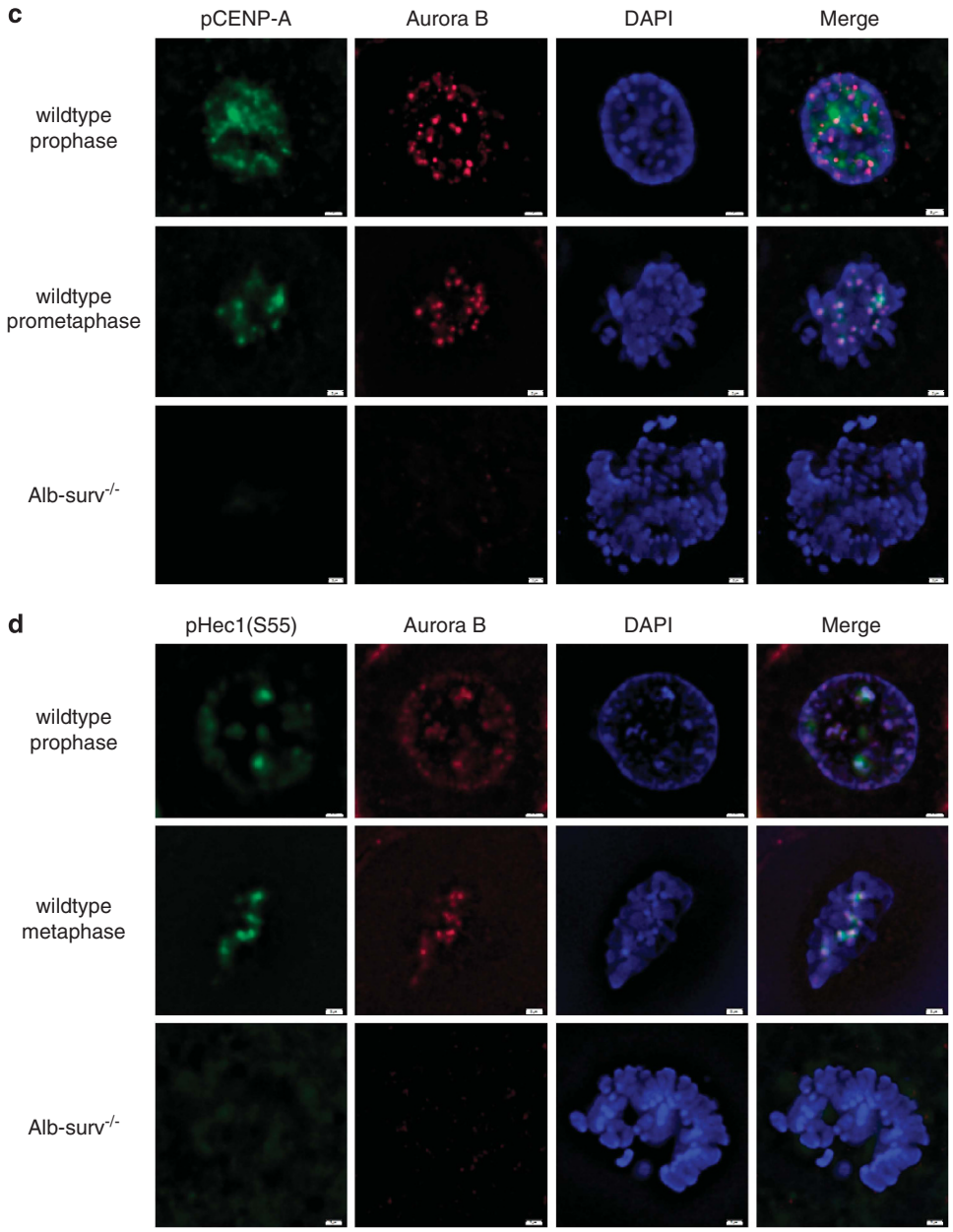

Figure 6 (Continued)

Progressive enlargement of nuclei and increasing polyploidy suggest a multiplication of DNA content and a failure to complete the cell cycle. The CPC has a role in chromosome segregation and in controlling the spindle assembly check point and in cytokinesis. The homolog of Survivin in *Saccharomyces cerevisiae* Bir1 has been shown to have a crucial role in the linkage of centromeres to microtubules. It has been proposed that this linkage is the tension sensor that relays the centromere–microtubule attachment into the local control of the Ipl1-kinase, the *Saccharomyces* homolog for Aurora B.[20] These mechanisms may explain the initially observed phenotype of Survivin-deficient hepatocytes with giant nuclei.

Following injury, the liver is a highly regenerative organ that is able to completely restore the loss of 70% of total liver mass. Recently Survivin was observed to have a role in liver regeneration in rodents and humans.[3,4] In regeneration experiments, we could show that the reduced hepatocyte number is likely due to decreased proliferation rather than increased cell death, as no differences in apoptosis were detected. Previous reports demonstrated that depletion of Survivin, inspite of leading to a major defect in mitosis, was not associated with an increase of apoptosis.[19,22] The data obtained in our study indicate that Survivin is necessary for proper hepatocyte proliferation and mitosis in development as well as in regeneration, especially for cell division following DNA replication. This is supported by the increased polyploidy of the hepatocytes and reduced total cell number, which was also seen in other gene knockout studies.[7,18,19] The increased

DNA content in Survivin-deficient hepatocytes does not induce apoptosis. Furthermore, loss of Survivin seems to be insufficient for disabling further rounds of DNA replication, as subpopulations of cells exhibited DNA contents up to 21*n*. Therefore, it can be concluded that Survivin is necessary for accurate hepatocyte cell division. The reason why a conditional knockout of Survivin leads to increased apoptosis only in some tissue types but not in others still remains enigmatic.[19,23,24]

Immunofluorescence staining of the CPC proteins Aurora B and INCENP in mitotic hepatocytes lacking Survivin indicated an apparent loss of these proteins. For Aurora B and INCENP, discrete signals were detectable in interphase nuclei of Alb-surv$^{-/-}$ hepatocytes and a diffuse cytoplasmic staining pattern for Aurora B. This is in contrast to the punctuated pattern in mitotic wild-type hepatocytes in which INCENP and Aurora B colocalized within the nucleus, which is in accordance with the current working hypothesis that Survivin is responsible for targeting the CPC to chromosomes. In previous studies, it was shown that Survivin interacts with Aurora B, the N-terminus of INCENP and Borealin to form the CPC, which is relocated from the kinetochore to the central spindle at the beginning of anaphase.[10,14] The observations of the different localization of the CPC and the absence of increased apoptosis suggest a role for Survivin in the context of mitosis rather than cell death in this hepatocyte-specific Survivin knockout model.

Survivin expression levels were decreased to 19% as compared with wildtype levels. Existing residual Survivin signal might be due to non-hepatocyte cell types in the liver remaining unaffected by the hepatocyte-specific Survivin knockout. Interestingly, Western blot analysis revealed a clear decrease in phosphorylated Histone H3(S28) in Survivin-deficient hepatocytes at day 3 of liver regeneration, whereas no changes were observed in phosphorylated Histone H3(S10) levels. Phosphorylation of Histone H3(S10) during mitosis is conserved in eukaryotes, well characterized throughout the cell cycle and used as a marker for mitotic cells. Different studies showed a close connection of phosphorylation of Histone H3(S10) and (S28) with chromosome condensation and also suggest an important role in chromosome segregation.[25–28] The reduced phosphorylation of Histone H3(S28) is therefore in line with the observed reduction of mitotic activity in combination with polyploidy and macronucleation in the regeneration experiments of Alb-surv$^{-/-}$ hepatocytes. A possible explanation for the decrease in Histone H3(S28)-phosphorylation is the reduced activity of Aurora B, which is required for phosphorylation of Histone H3(S28) during mitosis and for regulation of several additional mitotic processes.[29–31] It was shown that Survivin, Borealin and INCENP are required for activation and correct localization of Aurora B.[32] However, in Survivin-deficient hepatocytes, protein levels of Aurora B and INCENP seem to be unaffected despite deranged localization. Kinase assays showed an inadequate induction of Aurora B-activity during regeneration process in liver lysates lacking Survivin, compared with the wild-type lysates. Additionally, we could show that CENP-A and Hec1 – both centromeric substrates of Aurora B – were not phosphorylated in mitotic Alb-surv$^{-/-}$ hepatocytes. These data strongly suggest that the observed reduction of phosphorylated Histone H3(S28) in mitotic Survivin-deficient livers is a result of reduced Aurora B-activity. Recently it was shown that VRK-1 redundantly phosphorylates Histone H3(S10) but not Histone H3(S28).[33] Therefore, our finding of no changes in pHistone H3(S10) levels in regenerating Alb-surv$^{-/-}$ hepatocytes might be a result of alternative phosphorylation by VRK-1.

DeLuca *et al.*[34] proposed that phosphorylation of Hec1/Ndc80 serves as a control mechanism for the correct attachment of microtubules to the kinetochore by destabilization of the microtubule–kinetochore interaction leading to unattached kinetochores ready for reattachment by new microtubules. Our immunofluorescence stainings showed a complete lack of Hec1/Ndc80 phosphorylation on serine 55 in Alb-surv$^{-/-}$ hepatocytes, while in wild-type hepatocytes, a clear colocalization of Aurora B and pHec1(S55) in prophase and metaphase was present. In contrast to previous studies, we detected pHec1(S55) signals at the centromeres in metaphases and not at the spindle poles.[35] This discrepancy can be explained by the use of different antibodies.

An important substrate for Aurora B is the H3-related histone CENP-A. While CENP-A is essential for the recruitment of proteins to the kinetochore and providing a scaffold for the assembly of the functional kinetochore complex on the centromere,[36] the function of phosphorylation on Ser 7 during mitosis is not fully elucidated. It was shown that phosphorylation of CENP-A by Aurora B was strongest at incorrect microtubule attachments, and increased phosphorylation of kinetochore substrates leads to destabilization of kinetochore microtubules.[9] Therefore, dephosphorylation results in the stabilization of microtubule attachment at the kinetochore. Furthermore, inhibition of Aurora B, depletion or misplacement of the CPC caused an override of the spindle assembly checkpoint in the presence of mal-attached microtubules, but cells were not able to execute proper cytokinesis.[37] Targeting a cavity-induced allosteric site of Survivin led to disturbed alignment of the chromosomes on the metaphase plate resulting in apoptosis, without affecting overall DNA synthesis.[38] However, in our model, we did not observe increased apoptosis but increased DNA content in individual hepatocyte nuclei. In summary, these studies could provide a possible explanation for reduced mitotic activity in combination with increased polyploidy of Alb-surv$^{-/-}$ hepatocytes in our regeneration experiments. Caused by the absence of Survivin, Aurora B activity is reduced and the CPC itself is not properly located at the centromeres. The centromeric target proteins Histone H3, CENP-A and Hec1 are not phosphorylated, which in turn leads to stabilization of mal-attached microtubules at the kinetochores. As a result, the spindle checkpoint is passed without execution of proper cytokinesis.

In conclusion, mouse livers that lack Survivin exhibit macronucleation, polyploidy and reduced total hepatocyte number based on disturbed cell division without alterations in apoptosis. Lower levels of phosphorylated Histone H3(S28), CENP-A and Hec1(S55) are the result of the disturbed localization of the CPC and reduced Aurora B activity, leading to polyploid cells that do not execute proper cytokinesis. Here, we show for the first time the impact of survivin deletion on mitosis in a regenerating organ. Improved knowledge of the molecular functions of Survivin may lead to the development

of novel targeted drugs important for manipulating proliferation for cancer treatment or tissue regeneration.

Materials and Methods

Generation of mice with hepatocyte-specific deletion of survivin. Mice homozygous for a floxed survivin allele[7] were crossed with heterozygous mice that express Cre-recombinase under the control of the albumin promoter (alb-Cre; Jackson Laboratory, Sacramento, CA, USA). For genotyping of microdissected hepatocytes, deletion-specific PCR was performed. For single-cell laser microdissection, a PALM Robot-Microbeam (PALM GmbH, Bernried, Germany) was applied, and 100 hepatocytes or 150 interstitial cells were separately dissected and pooled.

70% liver resection for liver regeneration experiments. 70% liver resection was carried out by a trained microsurgeon under intraperitoneally administered ketamine/xylazine-anaesthesia (0.6–1.2 mg ketamine per gram of bodyweight; 0.1–0.2 mg xylazine per gram of bodyweight). Animals were sacrificed 12 h and at 1, 2, 3, 7 and 14 days after surgery. Analysis was performed with 3–5 animals per genotype and time point. All studies were performed with approval of the local Animal Ethics Committee.

Mean hepatocyte diameter and calculation of total hepatocyte number. Hepatocyte diameters were determined by measuring 100 hepatocytes on diastase-PAS stained sections using an image analyzing program (KS 300, Zeiss, Göttingen, Germany). To calculate the total number of hepatocytes, an established stereological method for quantification of cells per organ was used. Briefly, the volume fraction of hepatocytes (Vv Hep) in 10 randomly selected visual fields was determined by the principle of Delesse (area density = volume density). A grid containing 560 points was laid over the images and the points containing portal fields and blood vessels were counted. Vv Hep was calculated as a percentage. The mean hepatocyte volume was calculated as follows: $V\ Hep = \pi (\text{mean diameter}_{hepatocyte}/2)^2 \times \text{mean length}_{hepatocyte}$. The absolute number of hepatocytes (N Hep) was calculated as follows: N Hep = (Vv Hep × liver volume)/V Hep. Liver volume was calculated by dividing its weight by specific gravity (1.0048).

Quantification of Ki-67 and counting of mitotic figures. Specimens were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (3 μm) were pretreated with citrate buffer at pH 6.0 for 30 min at 98 °C and incubated with anti-KI-67 antibody (DCS Biogenex, Hamburg, Germany; dilution 1:50) for 60 min at room temperature. Detection was accomplished using donkey anti-rabbit-FITC antibody (Dianova, Hamburg, Germany; dilution 1:20) for 60 min at room temperature followed by 4′,6-diamidino-2-phenylindole (DAPI) counterstaining. For quantification, Ki-67-positive hepatocyte nuclei in 10 high-power fields were counted at a magnification of × 200. At least 3000 cells were included in the calculation. Mitotic figures were counted in sections stained with H&E.

Immunofluorescent staining. Briefly, specimens were fixed overnight in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections (1 μm) were pretreated with citrate buffer at pH 6.0 for 30 min at 98 °C. Primary antibodies used for immunofluorescence microscopy were: anti-Aurora B (BD Biosciences, Franklin Lakes, NJ, USA), anti-INCENP (Cell Signaling Technology, Danvers, MA, USA), anti-Survivin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pCENP-A (S7) (Upstate Biotechnology, Lake Placid, NY, USA), anti-pHistone H3 (S28) (Novus Biologicals, Littleton, CO, USA), anti-pHistone H3 (S10) (Millipore, Billerica, MA, USA) and anti-pHec1 (S55) (Biozol Diagnostica, Eching, Germany). Anti-Aurora B antibody was labeled with Alexa Fluor 555 (Life Technologies, Grand Island, NY, USA). Anti-INCENP, anti-Survivin, anti-pCENP-A (S7), anti-pHistone H3 (S28), anti-pHistone H3 (S10) and anti-pHec1 (S55) were labeled with Alexa Fluor 488 (Life Technologies). DNA was stained with DAPI and microscopy was carried out using an Olympus BX43 (Olympus Deutschland, Hamburg, Germany). Z-optical stacks with a spacing of 0.4 μm were recorded and deconvolution of images was carried out using the Olympus Cellsens imaging software. For further details see Supplementary Table 1 and Table 2.

Detection of apoptosis. Apoptosis was quantified by active caspase detection on snap-frozen tissue. Active caspase-3 staining was carried out using the Caspa-Tag *in-situ* detection kit (Chemicon, Temecula, CA, USA). For counterstaining 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Steinheim, Germany) was used. The percentage of CaspaTaq-positive nuclei was calculated in 10 visual fields using an image analysis system (KS 300, Zeiss, Oberkochen, Germany) counting at least 3000 cells.

Measurement of DNA content. DNA content was quantified by automated DNA cytometry. Nuclei were isolated using 50-μm thick paraffin sections treated with 0.5 mg/100 ml pepsin porcine in 0.07 M HCl for 30 min at 37 °C. Feulgen reaction was applied to nuclei and analyzed with DNA cytometry software (CYDOK, Fa Hilgers, Königswinter, Germany).

Western blotting. Lysates were prepared in lysis buffer (50 mM Tris, pH 8.0, 250 mM NaCl, 0.5% NP-40, 5 mM EDTA, 50 mM NaF, 50 mM Na_3VO_4, protease inihibitors), separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked with 5% skimmed milk/TBS pH 7.6/0.1% Tween20 or 5% skimmed milk/0.1 M NaCl/1 M Tris pH7.4/0.1%Tween 20 for 1 h at room temperature and incubated with antibodies against Survivin (Abcam, Cambridge, UK), Aurora B (Abcam), INCENP (Cell Signaling Technology), pHistone H3 (serine 10) (Millipore) and pHistone H3 (serine 28) (Novus Biologicals). Corresponding horseradish peroxidase-labeled anti-rabbit immunoglobulin G (1:10000; Cell Signaling Technology) was then added for 60 min at room temperature. Finally, enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) was used to visualize the results. Expression levels were analyzed by densitometry and normalized to GAPDH. Phosphorylated Histone H3 (S10, S28) levels were additionally normalized to mitotic numbers and average DNA content. For further details see Supplementary Table 3.

***In vitro* Aurora B kinase assay.** Snap-frozen tissues were lysed in 1 × TSE (50 mM Tris pH7.2, 300 mM NaCl, 5 mM EDTA, 0.01% NaN_3)/1 × IBX (50 mM NaF, 5 mM $Na_4P_2O_7$, 0.1 mM Na_3VO_4, 0.002% NaN_3)/1%TritonX-100/protease inhibitors (10 μg/ml soybean trypsin inhibitor, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride). Cell lysates (700 μg/sample) were precleared with Protein A/G sepharose beads (GE Healthcare Biosciences, Pittsburgh, PA, USA) (30 min, 4 °C). For immunoprecipitation of Aurora B, anti-Aurora B antibodies (3 μg/sample; Acris Antibodies, Herford, Germany) were added to cell lysates and rotated for 1 h at 4 °C. Protein A/G sepharose beads were then added to the lysates and samples were rotated for an additional hour. Beads were washed three to four times in a buffer containing 20 mM Hepes (pH7.2), 10 mM $MgCl_2$ and 2 mM EDTA. For the kinase assay myelin basic protein (MBP; 1 μg/sample; Sigma-Aldrich), 10 μM ATP and [γ-^{32}P]ATP (0.5–0.75 μCi/sample; Hartmann-Analytic, Braunschweig, Germany) were added to immunoprecipitated Aurora B kinase. After 30 min at 30 °C, the phosphorylation was stopped by addition of SDS-sample buffer. Unspecific kinase activity was determined by addition of the specific Aurora B kinase inhibitor ZM447439 (10 μM; Enzo Life Sciences, Loerrach, Germany) to the indicated cell lysates during immunoprecipitation, washing and kinase activity assay. After separation of the proteins, phosphorylated MBP was quantified by phosphoimager analysis.

Statistical analysis. Data are expressed as mean ± S.E. Statistical analysis was performed by using Mann–Whitney *U*-test and one-way ANOVA. A *P*-value < 0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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Authors contributions

Study design: BL, HAB, AM. Data acquisition: SH, SB, DM, GPP, GK, KL, BS. Material support: EMC, AM. Manuscript drafting: SH, JW, EMC, HAB. Important intellectual content: AM.

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