c-Myc regulates cell size and ploidy but is not essential for postnatal proliferation in liver

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The c-Myc protein is a transcription factor implicated in the regulation of multiple biological processes, including cell proliferation, cell growth, and apoptosis. *In vivo* overexpression of *c-myc* is linked to tumor development in a number of mouse models. Here, we show that perinatal inactivation of c-Myc in liver causes disorganized organ architecture, decreased hepatocyte size, and cell ploidy. Furthermore, c-Myc appears to have distinct roles in proliferation in liver. Thus, postnatal hepatocyte proliferation does not require c-Myc, whereas it is necessary for liver regeneration in adult mice. These results show novel physiological functions of *c-myc* in liver development and hepatocyte proliferation and growth.

The correct balance between cell proliferation and cell death is essential for the development of multicellular organisms. The Myc proteins are basic region/helix–loop–helix/leucine zipper transcription factors involved in the regulation of cellular proliferation, apoptosis, and cell growth (1, 2). The Myc family includes three closely related genes, c-, N-, L-Myc, which share similar biological activities, and all three have oncogenic potential. On this regard, L-Myc and N-Myc have been shown to be functionally equivalent to c-Myc-dependent activities in c-Myc nullizygous fibroblasts (3). Deregulated expression of *c-myc* is usually associated with the development of tumors in mice and humans (4, 5). C-Myc expression promotes the transition from G_0/G_1 to S phase of the cell cycle in multiple cell types, including hepatocytes, by regulating cyclin/cyclin-dependent kinase complexes (6–8). The G_0/G_1 to S-phase transition observed in hepatocytes of regenerating livers after partial hepatectomy (PH) correlates with rapid induction of *c-myc* and *n-myc* transcripts (9, 10). In limiting serum conditions, *c-myc* overexpression induces apoptosis in fibroblast and myeloid cell lines (11, 12). In hepatocyte cell lines, c-Myc expression causes apoptosis by inducing oxidative stress, and inhibition of c-Myc expression sensitizes hepatocytes to TNF-induced apoptosis (13, 14).

Transgenic mice overexpressing *c-myc* in liver show increased proliferation and apoptosis and develop tumors after a long latency period (15). More recently, *c-myc* has been shown to play an important role in the regulation of cell size in mice and flies (16, 17). In liver, hepatocytes can augment cell size by increasing cell ploidy through an altered cell cycle without cytokinesis (18). Interestingly, *c-myc* has been shown to regulate cell ploidy in hepatocytes from transgenic mice (19). Whether c-Myc regulates hepatocyte size is not clear. According to Trumpp *et al.* (20), c-Myc controls cell number instead of cell size in the organs of mice expressing hypomorphic alleles of *c-myc*. In this regard, a new role for *Drosophila* Myc has emerged in control of organ size by cell competition in flies (21, 22).

Germ-line inactivation of *c-myc* leads to multiple abnormalities and death at day 9–10 of embryonic development (23). Here, we used an inducible conditional approach to analyze the multiple functions of c-Myc in the context of liver growth in newborn and adult mice.

Materials and Methods

Mice. *c-myc*$^{fl/fl}$ conditional knockout mice (24) were bred with *mx-cre* mice (25), and progeny were bred to yield homozygous (*c-myc*$^{fl/fl}$;*mx-cre*$^{+}$) mice and control mice (*c-myc*$^{fl/+}$;*mx-cre*$^{+}$ and *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$). Mice were genotyped by using a PCR-based analysis of tail genomic DNA described in ref. 26. To amplify the *mx-cre* transgene (PCR product, 269 bp), primers SF-4 (5′-GCATAACCAGTGAAACAGCATT GCTG-3′) and 69R (5′-GGACATGTTCAGGGATCGCCAGGCG-3′) were used.

Polyinosinic-Polycytidylic Acid (pIpC) Injections. *C-myc* deletion was induced in newborns (day 2 after birth) by four i.p. injections (300 μg each) of pIpC (Sigma) at 2-day intervals. For *in vitro* hepatocytes cultures, newborn mice received four injections in consecutive days. For liver regeneration experiments, 6-week-old mice received four i.p. injections of pIpC (500 μg each) at 2-day intervals.

Isolation and Culture of Mouse Hepatocytes. Hepatocytes from 6- to 7-day-old mice were isolated by collagenase A treatment (0.4 mg/ml, Roche Diagnostics, Mannheim, Germany) and culture in poly-DL-lysine-coated plates (1 mg/ml, Sigma) in M199 medium (10% FCS/2.20% $NaHCO_3$/1 mM glutamine/120 μg/ml penicillin/100 μg/ml streptomycin), EGF (20 ng/ml, GIBCO), and insulin (10 nM, Sigma) for 24 h at 37°C in an atmosphere of 7.5% CO_2 (27). Hepatocytes were cultured alone or with L-ascorbic acid (1 mM, Sigma). In older mice (6–10 weeks), hepatocytes were isolated following perfusion with collagenase A. For cell-cycle analysis, treated cells were permeabilized and propidium iodide-stained (DNA-Prep reagent kit, Beckman Coulter) and incubated (30 min at 37°C) before FACS analysis.

Histology and Immunohistochemical Staining. All organs were paraffin-embedded and H&E-stained (4-μm sections). Staining for TUNEL assays followed manufacturer's protocols (TMRred *in situ* cell death kit, Roche Diagnostics). Samples were counterstained with DAPI (Vector Laboratories). For proliferating cell nuclear antigen (PCNA) staining, we used a biotinylated anti-human PCNA antibody (Pharmingen). Biotinylated antibodies were developed by using a StrepABComplex/horseradish

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Abbreviations: pIpC, polyinosinic-polycytidylic acid; PCNA, proliferating cell nuclear antigen; DCFH-DA, dichlorodihidrofluorescin diacetate; RPA, ribonuclease protection assay; PH, partial hepatectomy; H&E, hematoxylin/eosin.

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peroxidase kit. Horseradish peroxidase was visualized with diaminobenzidine (Sigma).

***In Vivo* BrdUrd Labeling.** The thymidine analogue BrdUrd (1 mg/ml, Sigma), prepared fresh every 3 days, was administered in drinking water for 1 week. BrdUrd staining was performed by using anti-BrdUrd antibody (Becton Dickinson) following the manufacturer's protocol.

Dichlorodihidrofluorescin Diacetate (DCFH-DA) Staining. Freshly isolated hepatocytes were incubated (45 min at 37°C) with the oxidative-sensitive probe DCFH-DA (5 μM, Molecular Probes).

PH. *c-myc* deletion was induced by using pIpC in adult mice (6 weeks old). At 48 h after PH (28), liver was analyzed by PCNA Western blotting.

Single-Cell PCR for *flox* and *null* Alleles. Single PCNA-positive hepatocytes were isolated from paraffin-embedded sections by using a laser dissection technique (UV microdissection). Genomic DNA from a single hepatocyte was obtained by 1 h incubation at 56°C in 20 μl of lysis buffer (1× PCR buffer/0.5 mg/ml proteinase K/9 μg/ml tRNA). Proteinase K was inactivated by heating (at 95°C for 10 min), and nested PCR was performed to amplify *flox* or *null* alleles independently. First-round PCR was carried out with half of the lysate (10 μl) and primers Myc-fl-1 (TGATATCGAATTCCTGCAGCC) and Myc-fl-3′ (TTTTCTTTCCGATTGCTGAC) to amplify *flox* alleles. Remaining lysate (10 μl) was used for first-round PCR to amplify *null* alleles with primers Myc-Δ-S (TCGCGCCCCTGAATTGCTAGGAA) and Myc-Δ-5.1 (TCCAGGATGCTAGAGACCTTCTCT). Second-round PCR was performed with 5 μl of the first-round reaction mixture an internal third primer, Myc-fl-2.1 (GTGTCAAATAATAAGAGACACCTCCCT) for *flox* alleles (fl-1 + fl-2.1, 100 bp), and Myc-Δ-2.1 (TTTAGGACATTTAGGTCGAGGGAC) for *null* alleles (Δ-S + Δ-2.1, 180 bp). PCR conditions were 94°C 5 min, followed by 40 cycles (94°C for 30 sec, 60°C 30 for sec, and 72°C for 30 sec) at 72°C for 7 min.

Ribonuclease Protection Assay (RPA). Total RNA was isolated from liver with the RNEasy kit (Qiagen, Hilden, Germany). The multiprobe Rnase protection assay kit was used (Becton Dickinson). Riboprobes of Myc family genes (BD Riboquant multiprobe template sets, BD Pharmingen, San Diego) were radiolabeled [$^{32}\alpha$]UTP using an *in vitro* transcription kit (BD Riboquant, BD Biosciences, San Diego) according to manufacturer's protocol. Total RNA (10 μg) was hybridized with radiolabeled probes. RNase treatment and gel resolution of protected probes were according to manufacturer's protocol. The gel was developed on Kodak X-AR film with intensifying screen at −80°C.

Results and Discussion

To circumvent the lethality of *c-myc* knockout mouse and inactivate *c-myc* gene in liver, we bred *c-myc*$^{fl/fl}$ mice (24) with inducible *mx-cre* transgenic mice (23, 25). F_1 generation intercrosses originated homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ and littermate control mice that express Cre recombinase in multiple tissues after induction of the *mx* promoter with IFN-γ or pIpC (25). Non-pIpC-injected *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ newborn and adult mice showed no evident phenotype compared to control littermates (data not shown).

To delete the *c-myc* gene, we injected pIpC i.p. into newborn mice, four times in the first 8 days after birth (see *Materials and Methods*). PIpC-injected *c-myc*$^{fl/+}$;*mx-cre*$^{+}$ mice, *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ mice, and wt control mice had no apparent phenotype difference (data not shown). PIpC-injected 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice showed reduced body weight compared with controls (Fig. 1*F*). After perinatal pIpC injection, 10-day-old and

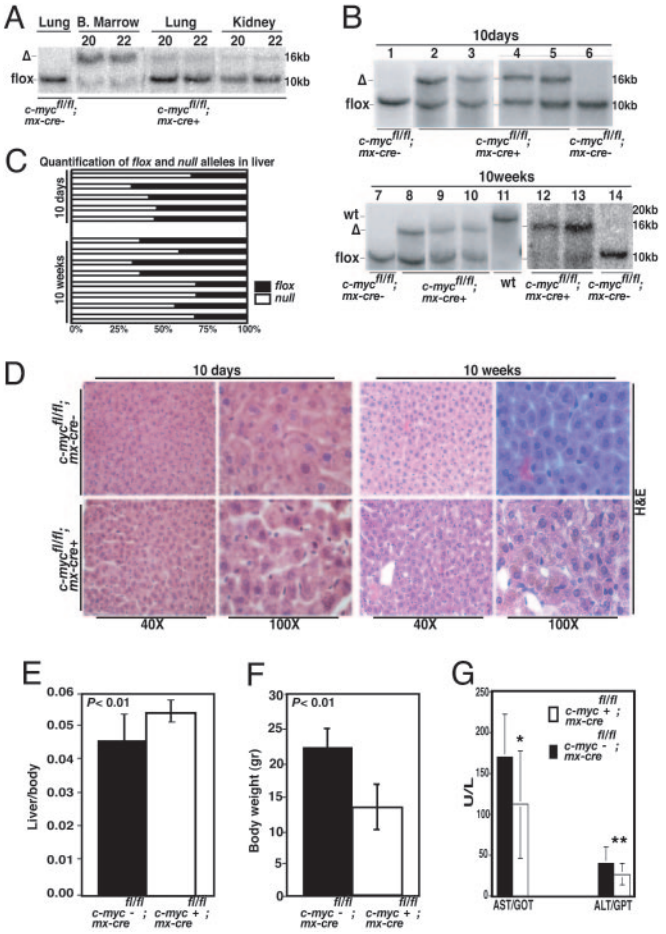

Fig. 1. Altered liver organization in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. (*A*) Deletion of *c-myc* gene in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Genomic DNA from the organs shown was isolated, *Eco*RI-digested, and probed as described (24). Two perinatally pIpC-injected 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice are shown. (*B*) Deletion of *c-myc* gene in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse liver. Genomic DNA from pIpC-injected 10 day-old and 10 week-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice was used. Southern blot was performed as in *A*. Numbers indicate individual mice. Genomic DNA from *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ mice or wt mice was used as a control. Experiment representative of multiple experiments. (*C*) Quantification of *flox* and *null* alleles in livers from pIpC-injected 10 day-old and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Quantification was performed with PhosphorImager, using IMAGEQUANT software on Southern blots performed as in *B*. %A allele, A allele counts × 100/total alleles counts (flox plus null counts). *A* is either *null* or *flox* alleles. (*D*) H&E-stained sections from 10 day-old and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ liver and control mouse liver. Seven 10-day-old mice of each genotype and seven 10-week-old mice of each genotype were analyzed. (*E*) Liver:body weight ratio of 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ and control mice. (*F*) Body weight of 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice from *E* (n = 30 for each genotype). (*G*) Hepatic function in 10-week-old mice. Serum levels of aspartate (AST/GOT) and alanine (ALT/GPT) aminotransferases. n = 14 for homozygous mice and n = 6 for control mice. U/L, units per liter. *, P = 0.0202; **, P = 0.0327 (Student's t test). In all experiments, mice were injected with pIpC as newborns (see *Materials and Methods*).

10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice showed minimal *c-myc* deletion (<10%) in kidney and lung (Fig. 1*A* and data not shown). Histology sections from these organs did not reveal any significant difference between homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control littermates (Fig. 5, which is published as supporting information on the PNAS web site). In contrast, efficient deletion of *c-myc* in bone marrow (≅90–100%) caused severe anemia in adult homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and death after 3 months

(Fig. 1*A*, data not shown, and E.B. and I.M.d.A., unpublished work).

After perinatal pIpC injection, livers from young (10 days old) and adult (10 weeks old) homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice showed efficient *c-myc* gene deletion (Fig. 1 *B* and *C*). The extent of *c-myc* deletion in liver (≅40–75%) was different among homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice (Fig. 1 *B* and *C*). These values were underestimated because of the differential transfer of *null* (Δ, 16 kb) vs. *flox* (10 kb) bands in Southern blots (data not shown). For our analysis in liver, we used mice that had 50% or more of *c-myc*-deleted alleles.

To determine whether *c-myc* gene deletion in liver affected parenchymal organization, we prepared hematoxylin/eosin (H&E)-stained sections from pIpC-injected 10-day-old and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice. Liver sections showed disorganized parenchyma, hepatocytes with reduced cell area, and a greater incidence of pyknotic nuclei in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse hepatocytes compared with those of controls (Fig. 1*D*). These defects in liver organization correlated with impaired hepatic function in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice when compared with control mice (Fig. 1*G*). These results suggest that perinatal *c-myc* deletion in liver severely affects parenchymal organization and function in liver of homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice.

H&E-stained sections indicated that hepatocyte size might be altered in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Overexpression of *c-myc* in B lymphocytes and hepatocytes increases cell size in mice (15, 17, 29). Accordingly, *Drosophila* Myc mutants have smaller cell size (16). In contrast, hepatocytes from mice harboring hypomorphic alleles of *c-myc* show normal cell size (20). To analyze whether *c-myc* affected hepatocyte size, hepatocytes were isolated from pIpC-injected homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice and analyzed by FACS. Forward scatter in FACS analysis showed a decrease in hepatocyte size in 10-day-old and 10-week-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with control littermates (Fig. 2*A* and data not shown). We also observed that adult *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse hepatocytes had a smaller cell area than controls (Fig. 2*B*). Furthermore, adult *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ livers contained more hepatocytes per field (201.5 ± 22.8) than those of control littermates (135.8 ± 12.7) (Fig. 2*C*).

Acquisition of polyploidy by hepatocytes is an age-dependent differentiation process characteristic of liver growth. At birth, the majority of hepatocytes are diploid mononucleated cells that cycle normally. Subsequently, hepatocytes undergo a modified cell cycle without cytokinesis and generate binucleated polyploids cells (18). c-Myc regulates cell ploidy in human primary keratinocytes, mouse hepatocytes, and flies (19, 30–32). To determine whether the lack of c-Myc affected cell ploidy in liver, we isolated primary hepatocytes from pIpC-injected 10-week-old mice and stained them with propidium iodide to measure DNA content. Polyploid hepatocyte populations can be identified by the side-scatter parameter (Fig. 2*D*, gate R1). The polyploid population was reduced in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with controls (Fig. 2 *D* and *E*). To see whether a decrease in the polyploid population correlated with a reduction in the number of binucleated cells in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with control mice, we performed binucleated cell countings in H&E-stained liver sections. We observed a decrease in the number of binucleated hepatocytes in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with control mice (Fig. 2*F*). These results indicate that c-Myc is necessary for normal acquisition of polyploidy in hepatocytes.

c-Myc is induced in proliferating cultured primary hepatocytes, and promotes progression from G_0/G_1 to S phase (8). To test whether c-Myc affects hepatocyte proliferation, we measured BrdUrd incorporation in liver sections of pIpC-injected 6- and 10-week-old mice. We observed a larger number of proliferating BrdUrd^{+} hepatocytes in the liver of 6-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice than in control mice (Fig. 3*A* and Table 1). To determine whether the *c-myc* gene in proliferating hepatocytes was intact, nondeleted (*flox*/*flox*) or deleted (*null*/*null*), we performed a genomic PCR assay on single PCNA-positive cells isolated by laser (UV microdissection) from liver sections of homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. PCNA was used to select proliferating cells because genomic DNA from BrdUrd^{+} hepatocytes is not amplified by PCR (data not shown). Cells that gave a PCR product corresponding to the *c-myc*-deleted gene did not amplified a *flox* allele and vice versa. Therefore, this analysis identified cells with the *c-myc* gene deleted (Fig. 3*B*). These results show that hepatocytes are at least capable of entering the S phase of the cell cycle in the absence of *c-myc*. These analysis cannot rule out the possibility that BrdUrd^{+} or PCNA^{+} cells do not complete S phases. However, the fact that the ratio between deleted (null) and nondeleted (flox) hepatocytes in 10-day-old and 10-week-old mice is maintained supports the notion that c-Myc-deficient hepatocytes do

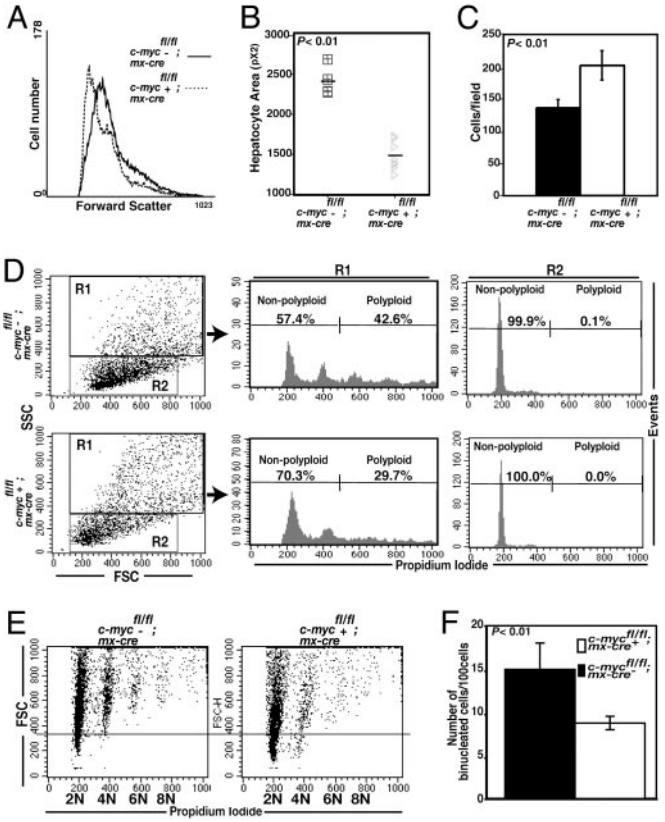

Fig. 2. Decreased cell size and ploidy in hepatocytes from *c-myc*$^{fl/fl}$; *mx-cre*$^{+}$ *mice*. (*A*) Reduced hepatocyte size in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Hepatocytes from 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ control mice were isolated and analyzed by FACS. A total of four mice of each genotype were analyzed. (*B*) Area of hepatocytes from H&E-stained liver sections of 10-week-old mice (IMAGEJ29 software). pX^2, square pixels. (*C*) Hepatocyte number per field (two fields per mouse) in liver sections from mice as in Fig. 1*D*. For cell area and hepatocytes per field, five control mice and nine homozygous mice were analyzed. (*D*) Decreased cell ploidy in hepatocytes from 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Side-scatter plot of whole populations is shown. R1 gate is used to discriminate polyploid cells; R2 gate contains nonpolyploid cells only. Numbers represent the percentage of cells in each gate within each plot. (*E*) Cell size vs. DNA content (propidium iodide). Hepatocytes from mice of indicated genotypes were isolated and analyzed by FACS. All mice were injected with pIpC as newborns. A line has been drawn to compare FSC values. Example representative of three independent experiments. (*F*) Reduced number of binucleated cells in liver from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. The number of binucleated cells per field were counted (three fields per mouse), and the number was normalized by 100 cells per field. The graph shows the mean of four mice for each genotype. *P* value was determined by using Student's *t* test.

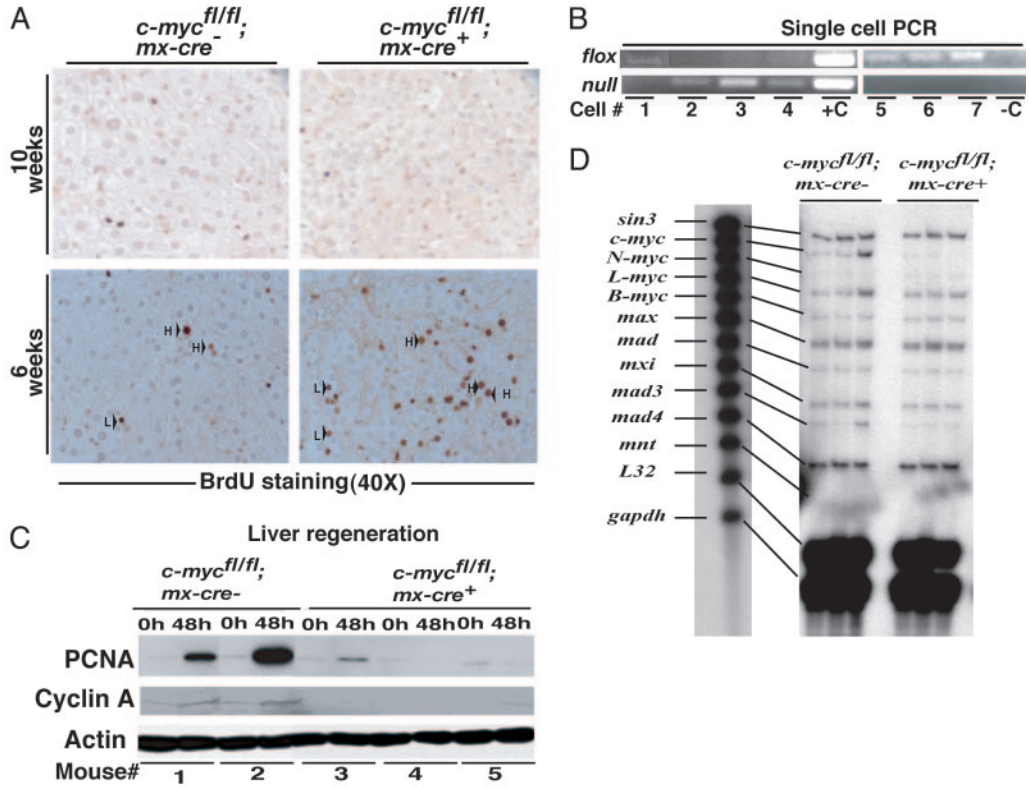

Fig. 3. Hepatocyte proliferation in the absence of c-Myc. (*A*) Proliferation of hepatocytes from 6- and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and *c-myc*$^{fl/fl}$;*mx-cre*$^{-}$ control mice. BrdUrd was added to drinking water for 1 week. Liver sections were prepared and stained with an anti-BrdUrd antibody. Arrows indicate hepatocytes (H) or lymphocytes (L). Two control mice and three 6- or 10-week-old homozygous mice were analyzed. (*B*) Genomic PCR of single PCNA^{+} cells from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. Liver sections were prepared and stained with anti-PCNA antibody. Single PCNA^{+} cells were isolated by laser dissection. Genomic DNA from each single PCNA^{+} cell was PCR-amplified by using *flox* (nondeleted)- and *null* (deleted)-specific primers (see *Materials and Methods*). Genomic DNA from tail was used as a positive control (+C). Eleven PCNA^{+} cells from three homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice were analyzed in two independent experiments. (*C*) Impaired liver regeneration in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice injected as adults. Six-week-old mice were injected with pIpC. PH was performed; 48 h later, mice were killed and protein from liver was prepared. PCNA and cyclin A were measured by Western blot. Actin was used as a loading control. Three homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and two control mice were analyzed. (*D*) RPA with total RNA (10 μg) from liver of 10-day-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice. Three mice of each genotype are shown. Experiment representative of two independent experiments. In all experiments, except for the experiment with liver regeneration, mice were injected with pIpC as newborns.

not have a major proliferative disadvantage compared with nondeleted hepatocytes (Fig. 1 *B* and *C*).

To explore whether hepatocyte proliferation in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice might be due to a possible compensatory effect by other *myc* or *myc*-related genes, we used an RPA. RPA analysis showed no significant changes in gene expression of other *myc* or *myc*-related genes (Fig. 3*D*). Finally, redundancy of other *myc* genes vs. compensatory changes in gene expression could be responsible for the proliferation observed in c-Myc-deficient hepatocytes (3, 33).

Table 1. Quantification of BrdUrd^{+} hepatocytes

Genotype	Total no., three fields	BrdUrd^{+}, three fields	Percent BrdUrd^{+}
	10 weeks		
c-myc$^{fl/fl}$;*mx-cre*$^{-}$	338	3	0.8
c-myc$^{fl/fl}$;*mx-cre*$^{+}$	448	5	1.1
	6 weeks		
c-myc$^{fl/fl}$;*mx-cre*$^{-}$	321	6	1.8
c-myc$^{fl/fl}$;*mx-cre*$^{-}$	408	7	1.7
c-myc$^{fl/fl}$;*mx-cre*$^{+}$	480	28	5.8
c-myc$^{fl/fl}$;*mx-cre*$^{+}$	510	15	2.9
c-myc$^{fl/fl}$;*mx-cre*$^{+}$	473	17	3.5

BrdUrd^{+} hepatocytes were counted from sections shown in Fig. 3*A*.

To restore liver mass after PH, quiescent hepatocytes must undergo a G_0-to-G_1 transition and enter S phase (34); the *c-myc* and *n-myc* genes are rapidly induced after PH (10, 35, 36). We therefore analyzed whether liver regeneration was affected in pIpC-injected *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice that had been injected with pIpC as newborns did not survive the surgical procedure, which was likely due to anemia and reduced weight (data not shown); we thus performed PH in 6-week-old mice injected with pIpC 1 week before surgery (28). To monitor hepatocyte proliferation, we analyzed PCNA protein levels in livers of homozygous and control mice at 0 and 48 h after PH. Western blot showed decreased PCNA levels in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse liver compared with controls (Fig. 3*C*). Because PCNA has been reported as a c-Myc-responsive gene by using microarrays, we validated our results by looking at cyclin A protein levels by Western blot (37) (Fig. 3*C*). Based on these results, we conclude that liver regeneration is impaired in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice.

The liver damage observed in H&E-stained sections from pIpC-injected homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice lead us to investigate whether apoptosis was altered in these mice by TUNEL assays (Figs. 1*C*, 4*A*, and Table 2) (13, 14). We observed a higher incidence of apoptotic nuclei in livers from 10-day-old

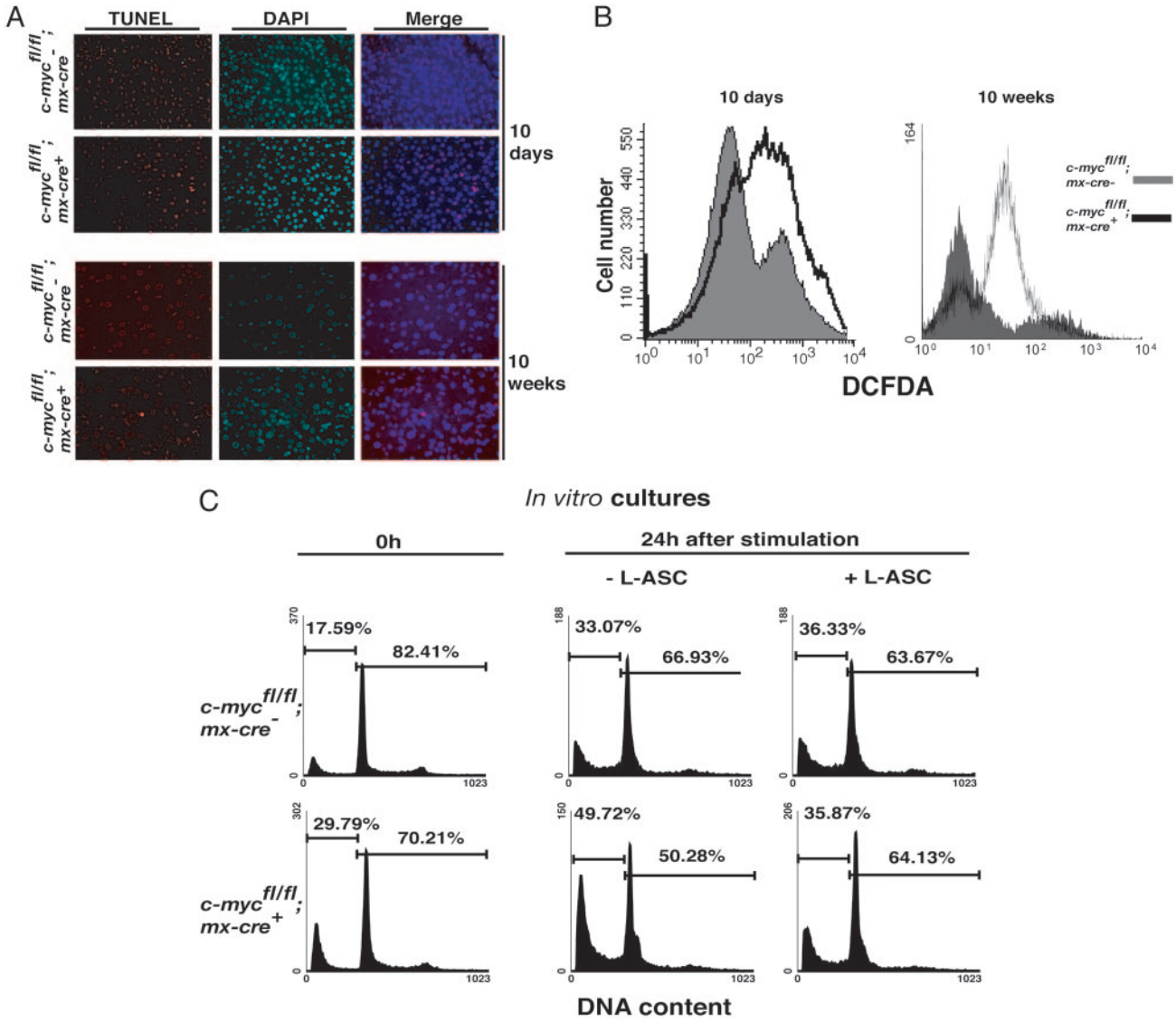

Fig. 4. Apoptosis in liver from *c-myc* $^{fl/fl}$;*mx-cre*$^{+}$. (*A*) TUNEL assays in liver sections from homozygous *c-myc* $^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice. (*B*) Increased free-radical content in homozygous *c-myc* $^{fl/fl}$;*mx-cre*$^{+}$ mouse liver. Hepatocytes from 10-day-old and 10-week-old homozygous and control mice were isolated and stained with the oxidation-sensitive probe DCFH-DA; a total of four mice of each genotype were analyzed. (*C*) Hepatocyte apoptosis is inhibited by the antioxidant L-ascorbic acid (L-Asc). Hepatocytes from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice and control mice were isolated and cultured *in vitro* alone or with L-Asc for 24 h. Cells were harvested at times indicated, propidium iodide-stained to measure DNA content, and subG$_0$/G$_1$ peaks (dead cells) were analyzed by FACS. Experiment representative of three independent experiments. In all experiments, mice were injected with pIpC as newborns.

and 10-week-old homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice compared with controls (Fig. 4*A* and Table 2). Increase in free radicals content has been shown to precede apoptosis in hepatocytes and in mouse models of experimental liver injury (38, 39). In human fibroblasts, c-Myc can induce DNA damage by a mechanism mediated reactive oxygen species (40). To test whether the cell oxidative status in *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse liver was affected, we analyzed primary hepatocytes from pIpC-induced mice with the oxidation-sensitive probe DCFH-DA. Ten-day-old and 10-week-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mouse hepatocytes showed a notable increase in DCFH-DA signal compared with control littermates (Fig. 4*B*).

To see whether increased apoptosis was mediated by free radicals, we treated activated primary hepatocytes from pIpC-injected 10-day-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice with the antioxidant ascorbic acid. We observed that primary hepatocytes from 10-day-old *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice were more sensitive to apoptosis during the isolation procedure than control hepatocytes (Fig. 4*C*, 0 h, and data not shown). Furthermore, we found inhibition of apoptosis in hepatocytes from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice cultured in the presence of ascorbic acid to levels comparable with control cells (Fig. 4*C*). Apoptosis in hepatocytes from *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice is thus mediated by free radicals, and can be inhibited by ascorbic acid.

We show that perinatal deletion of *c-myc* severely affects liver organization and function in homozygous *c-myc*$^{fl/fl}$;*mx-cre*$^{+}$ mice. In contrast to Trumpp *et al.* (20), we observe that *c-myc* can regulate cell size and number in liver. This difference could be explained by the intrinsic nature of the system used (hypomorphic vs. null *c-myc* alleles).

The capacity of c-Myc-deficient hepatocytes to proliferate might reflect the ability of hepatic stem cells to respond to the environmental factors present in each developmental stage (Fig. 3*A* and Table 1). It is possible that the effects observed in cell

Table 2. Quantification of TUNEL$^+$ hepatocytes

Mouse genotype	Cells per field	TUNEL$^+$, four fields	TUNEL$^+$, 100 cells
	10 days		
c-myc$^{fl/+}$;mx-cre$^-$	145	2	0.34
c-myc$^{fl/+}$;mx-cre$^-$	107	3	0.70
c-myc$^{fl/fl}$;mx-cre$^-$	121	1	0.21
c-myc$^{fl/fl}$;mx-cre$^+$	178	7	0.98
c-myc$^{fl/fl}$;mx-cre$^+$	169	6	0.89
c-myc$^{fl/fl}$;mx-cre$^+$	179	4	0.56
	10 weeks		
c-myc$^{fl/+}$;mx-cre$^-$	149	0	0
c-myc$^{fl/+}$;mx-cre$^-$	118	1	0.21
c-myc$^{fl/fl}$;mx-cre$^-$	145	0	0
c-myc$^{fl/fl}$;mx-cre$^-$	139	1	0.18
c-myc$^{fl/fl}$;mx-cre$^+$	210	3	0.36
c-myc$^{fl/fl}$;mx-cre$^+$	178	2	0.28
c-myc$^{fl/fl}$;mx-cre$^+$	230	1	0.12
c-myc$^{fl/fl}$;mx-cre$^+$	179	4	0.56
c-myc$^{fl/fl}$;mx-cre$^+$	223	0	0
c-myc$^{fl/fl}$;mx-cre$^+$	221	1	0.11

TUNEL$^+$ hepatocytes were counted from sections shown in Fig. 4*A*.

proliferation in livers of young and adult *c-myc$^{fl/fl}$;mx-cre$^+$* mice are directly related to the abundance of hepatic stem cells. Hepatic stem cells could respond to liver damage, decrease cell size, or surrounding cells with different c-Myc levels, by proliferating and differentiating in a c-Myc-independent manner in young mice. As mice get older, these hepatic stem cells either accumulate, lose the capacity to differentiate, or die by apoptosis. On this regard, c-Myc-deficient hematopoietic stem cells have been shown to proliferate and accumulate over time in the bone marrow of adult mice (41). Alternatively, liver cells seem to be very sensitive to changes in c-Myc expression. Mice overexpressing an inducible *c-myc* transgene in liver seem to expose the multipotency of hepatic stem cells to generate tumors, differentiate, or die, depending on the levels of c-Myc (42). Moreover, the response of liver cells to *c-myc* overexpression in liver is tightly linked to the developmental context (43). In the case of liver regeneration after PH, we observed that c-Myc is required for normal regeneration. This apparent contradiction might be explained by the time at which *c-myc* deletion is induced (newborn vs. adult). In adult mice, hepatocytes are quiescent (G_0) when *c-myc* deletion is induced and PH is performed; in this case, hepatocytes may require c-Myc to exit G_0. In contrast, after perinatal *c-myc* deletion, hepatocytes might not exit the cell cycle to enter the quiescence state (G_0) compared with control littermates (Fig. 3 *A* and *C*).

Homozygous *c-myc$^{fl/fl}$;mx-cre$^+$* mice showed reduced body size, and a slight increase in liver:body ratio compared with controls (Fig. 1 *E* and *F*). The mechanism(s) underlying the regulation of body and organ size are complex and beyond the scope of this paper. However, recent reports implicate *Drosophila* Myc in the regulation of organ size by cell competition in *Drosophila* imaginal discs (21, 22). We cannot rule out that the apoptosis observed in the liver of *c-myc$^{fl/fl}$;mx-cre$^+$* mice is due to wt cells inducing cell death of c-Myc-deficient cells as observed in *Drosophila* (21, 22). However, the presence of c-Myc-deficient hepatocytes in those mice that harbor extensive *c-myc* deletion 2 months after induction does not favor this possibility (Fig. 1*B*). Further studies are required that directly address these questions. On this regard, *c-myc$^{fl/fl}$;mx-cre$^+$* mice should be a useful model.

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