Supplemental information

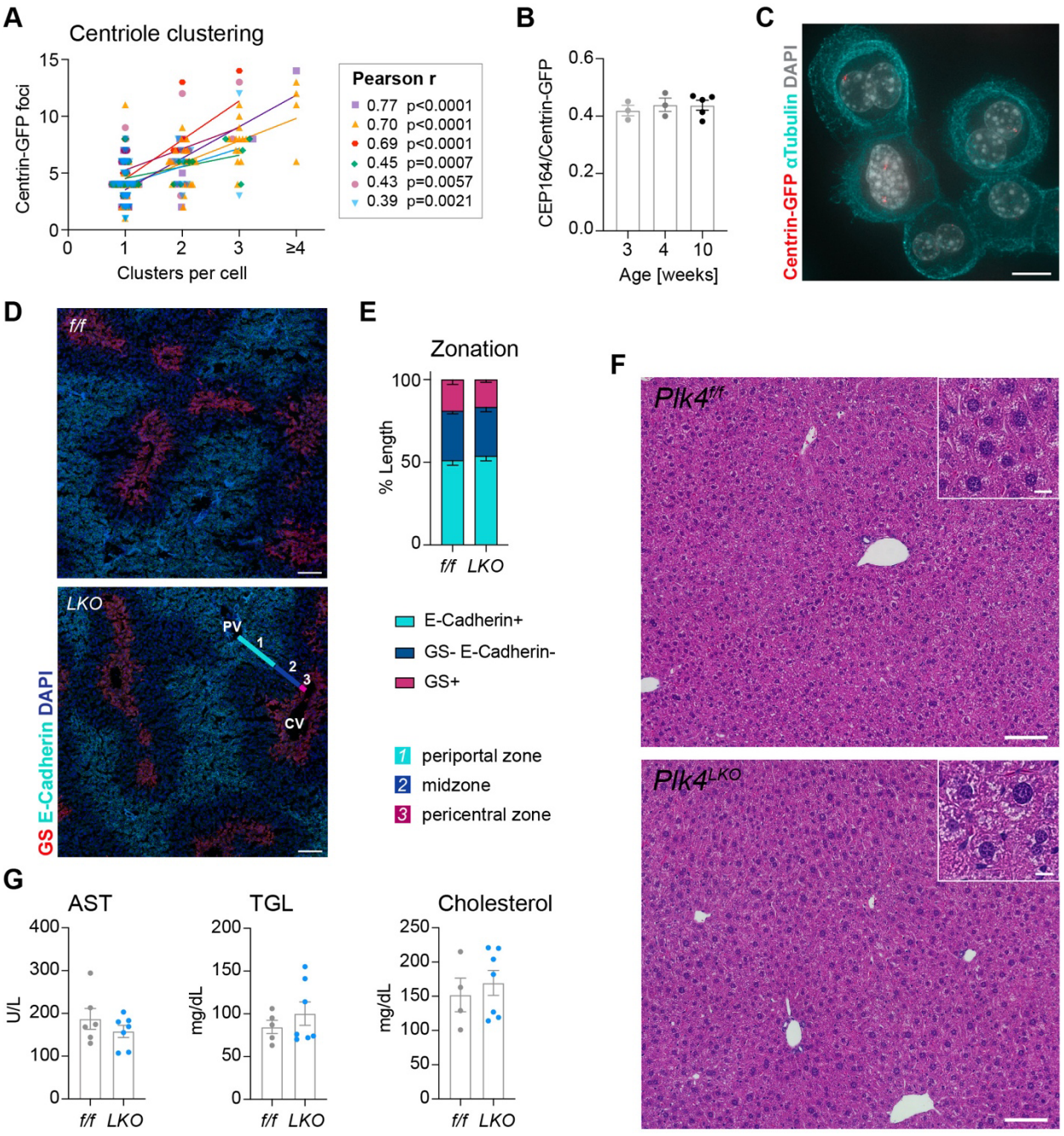
Centriole signaling restricts hepatocyte ploidy to maintain liver integrity

Valentina C. Sladky[1]*, Hanan Akbari[1], Daniel Tapias-Gomez[1], Lauren T. Evans[1], Chelsea G. Drown[1], Margaret A. Strong[1], Gina M. LoMastro[1], Tatianna Larman[2], Andrew J. Holland[1]*

[1]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

[2]Divison of Gastrointestinal and Liver Pathology, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

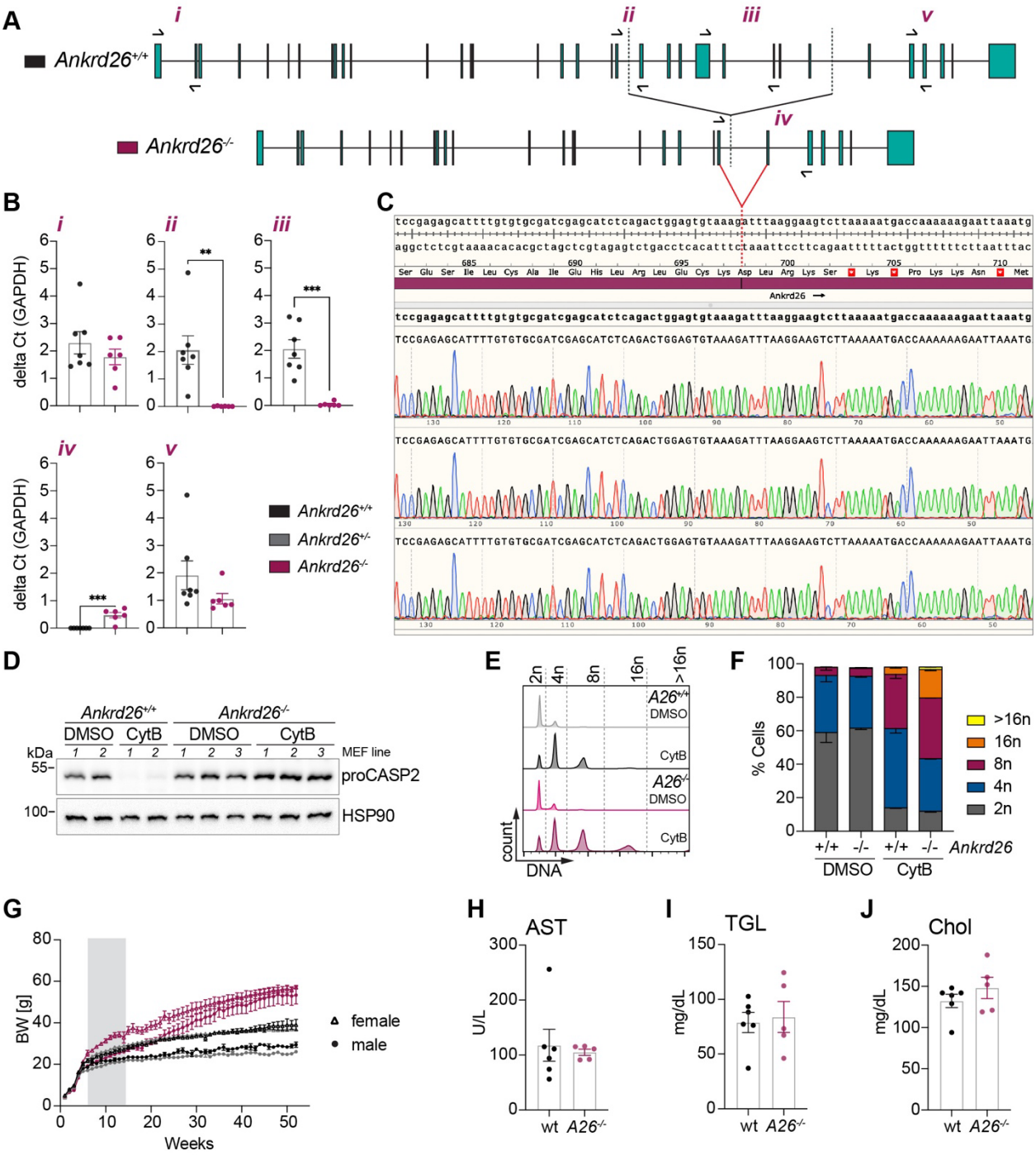
* Corresponding authors: vsladky1@jhmi.edu, aholland@jhmi.edu



Supplemental Figure 1: Centriole loss does not impact basal liver function

(A) Graph showing the correlation between the number of Centrin-GFP foci and the number of spindle poles for each genotype. The number of Centrin-GFP foci correlates with the number of centriole clusters with a Pearson R ranging from 0.39 to 0.77 (p 0.0021-<0.0001). Colored dots represent individual cells from a single mouse. Lines represent linear regression for each mouse.

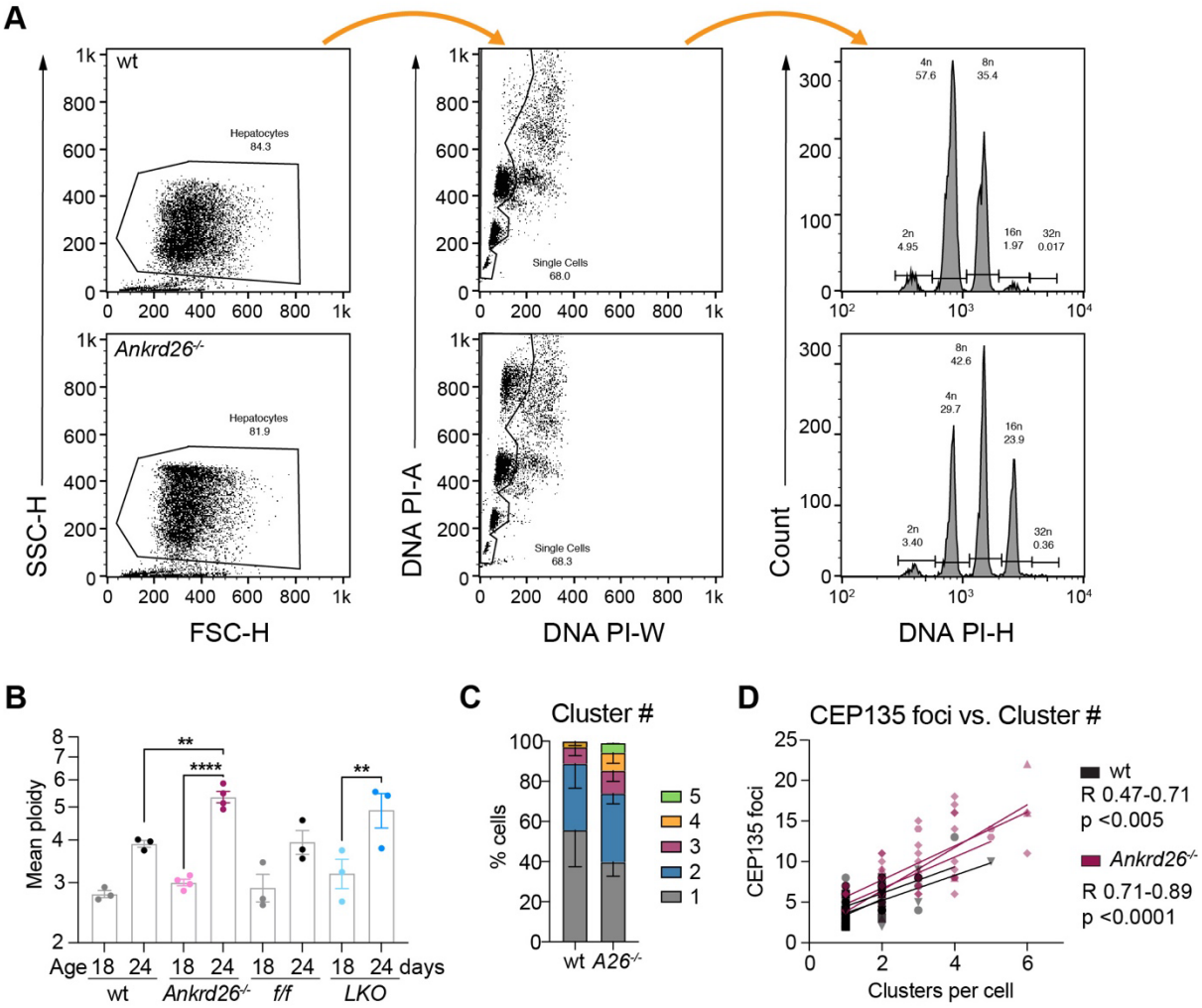
N=6 mice, n=40-65 cells per mouse. **(B)** The fraction of mature centrioles analyzed by immunostaining of isolated *Centrin-GFP$^{tg/tg}$* hepatocytes at 3, 4, and 10 weeks of age. Ages 3 and 4 weeks: N=3 mice, n=20-50 cells. Age 10 weeks: N=5 mice, n=40-120 cells. **(C)** Hepatocytes isolated from *Centrin-GFP$^{tg/tg}$* mice were attached to collagen-coated coverslips and immunostained for α-tubulin (cyan). Centrin-GFP is shown in red. **(D)** Liver zonation was determined by immunostaining for the periportal marker E-cadherin (cyan; zone 1) and the pericentral marker glutamine synthetase (GS, red; zone 3). Areas negative for E-cadherin and GS represent the midzone (zone 2). **(E)** Zonation was quantified as % distance between the portal vein (PV) and the central vein (CV), as indicated in (D). n=18-52 liver lobules were analyzed in N=3 *Plk4$^{f/f}$* and N=4 *Plk4^{LKO}* mice. **(F)** H&E stained paraffin section shows a tissue overview for *Plk4$^{f/f}$; Centrin-GFP$^{tg/tg}$* (f/f) and *Plk4^{LKO}; Centrin-GFP$^{tg/tg}$* (LKO) livers. Scale bar represents 100 µm for the overview images and 10 µm for the insets. **(G)** Serum concentrations of aspartate transaminase (AST) triglycerides (TGL), and cholesterol in 10 weeks old *Plk4$^{f/f}$; Centrin-GFP$^{tg/tg}$* (f/f) and *Plk4^{LKO}; Centrin-GFP$^{tg/tg}$* (LKO) mice. All data are represented as mean ± S.E.M. Statistical significance was assessed using an unpaired two-tailed Student's t-test (G), one-way-ANOVA with Sidak's multiple comparisons test (B), or two-way-ANOVA with Sidak's multiple comparisons test (E). Only significant differences are indicated.



Supplemental Figure 2: *Ankrd26*$^{-/-}$ animals show increased liver ploidy.

(A) Exon structure of the *Ankrd26* wt locus (+/+, black) and the *Ankrd26*$^{-/-}$ allele (purple) after deletion of exons 23-30. **(B)** qRT-PCR on total liver samples (wt N=7, *Ankrd26*$^{-/-}$ N=6) was used to determine the mRNA expression of different regions as indicated by primers (arrows) and

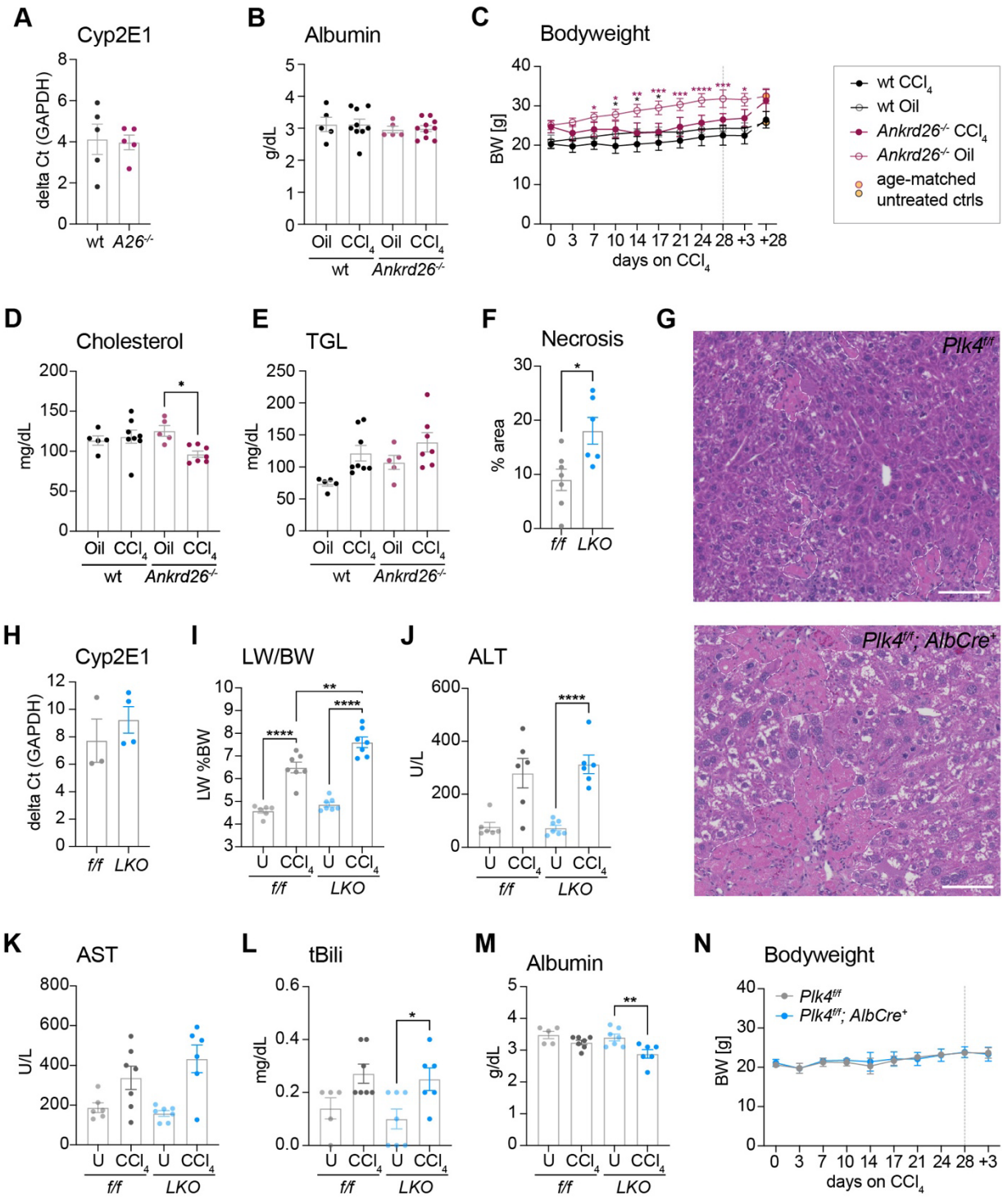
numbers (i-v) in (A). Expression is shown relative to GAPDH. **(C)** Sequencing of cDNAs generated from the liver of 3 *Ankrd26*$^{-/-}$ mice showing the splicing and junction of exons 22 and 31. This creates a frame-shift and premature stop codon after S703. **(D-F)** *Ankrd26*$^{+/+}$ and *Ankrd26*$^{-/-}$ MEFs were treated with DMSO or Cytochalasin B for 48h to induce cytokinesis failure. **(D)** Immunoblots were probed for pro-CASP2, and HSP90 serves as a loading control. **(E)** Representative flow cytometry histograms for (F). **(F)** Quantified ploidy distributions from MEFs of the indicated genotype. N=2 *Ankrd26*$^{+/+}$ and N=3 *Ankrd26*$^{-/-}$ MEF lines were analyzed. **(G)** *Ankrd26*$^{+/+}$, *Ankrd26*$^{+/-}$ and *Ankrd26*$^{-/-}$ mice were weighed weekly over 12 months. *Ankrd26*$^{+/+}$ female: N=4, male: N= 2. *Ankrd26*$^{+/-}$ female: N=3, male: N=9. *Ankrd26*$^{-/-}$ female: N=5, male: N=3. Grey box indicates the approximate age range of mice used in the experiments in figures 3-5. **(G)** Serum concentrations of aspartate transaminase (AST), **(H)** triglycerides (TGL), and cholesterol analyzed in 10 weeks old C57BL/6J (wt, N=6) and *Ankrd26*$^{-/-}$ mice (N=5). All data are represented as mean ± S.E.M. Statistical significance was assessed using an unpaired two-tailed Student's t-test (B, H-J). Only significant results are indicated. ** $p<0.01$, *** $p<0.001$.



Supplemental Figure 3: Centrioles cluster in *Ankrd26*$^{-/-}$ livers.

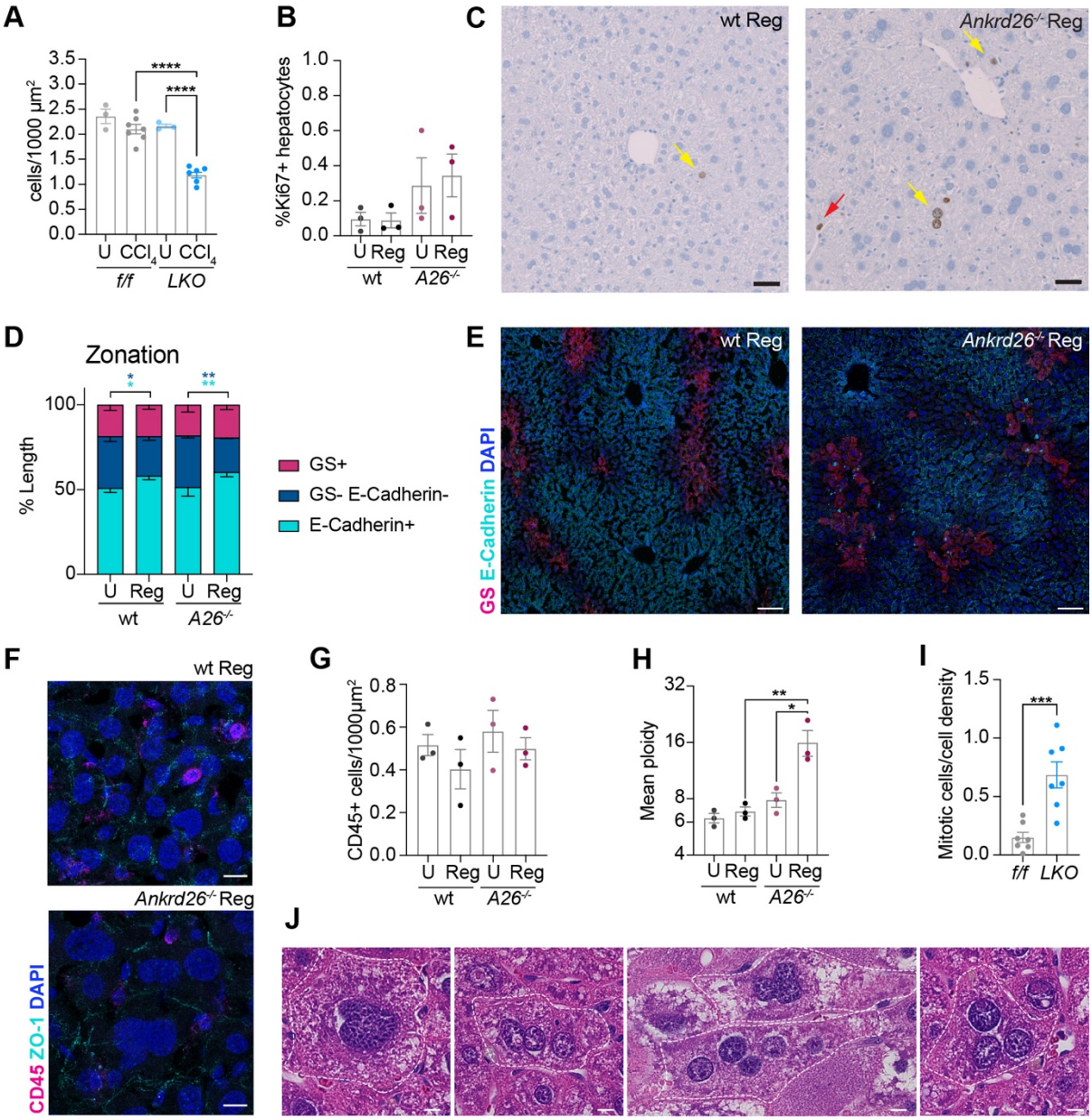
(A) Gating strategy for flow cytometric ploidy analysis. Cells were identified by FSC vs. SSC (forward scatter; sideward scatter), doublets were excluded based on propidium iodide signal width vs. area (DNA PI-W, A), and signal height (DNA PI-H) was plotted as histogram to assess the DNA distribution. Examples show 10 weeks old wt and *Ankrd26*$^{-/-}$ mice. **(B)** Mean ploidy of hepatocytes isolated at 18 days or 24 days of age. wt 18d N=3, 24d N=3; *Ankrd26*$^{-/-}$ 18d N=4, 24d N=3; *Plk4*$^{f/f}$*; Centrin-GFP*$^{tg/tg}$ (f/f) 18d N=3, 24d N=3; *Plk4*^{LKO}*; Centrin-GFP*$^{tg/tg}$ (LKO) 18d N=3, 24d N=3. The data for wt and *Ankrd26*$^{-/-}$ mice are the same as shown in Fig 2D and included here for comparison. **(C)** Percentage of cells with different numbers of centriole clusters assessed by counting CEP135 foci in immunostained wt and *Ankrd26*$^{-/-}$ liver sections, as shown in Fig 2L. **(D)** Graph showing the correlation of centriole number with the number of clusters per cell analyzed for wt (Pearson R=0.47-0.71, p<0.005) and *Ankrd26*$^{-/-}$ (Pearson R=0.71-0.89, p>0.0001) livers.

Lines represent a linear regression calculated per mouse. N=3 mice per genotype, n=20-60 cells per mouse for (C-D). Data are represented as mean ± S.E.M. Statistical significance was determined using one-way-ANOVA with Sidak's multiple comparisons test (B). Only significant results are indicated. ** $p<0.01$, **** $p<0.0001$.



Supplemental Figure 4: ANKRD26-deficiency or loss of *Plk4* promotes CCl4-induced chronic liver injury.

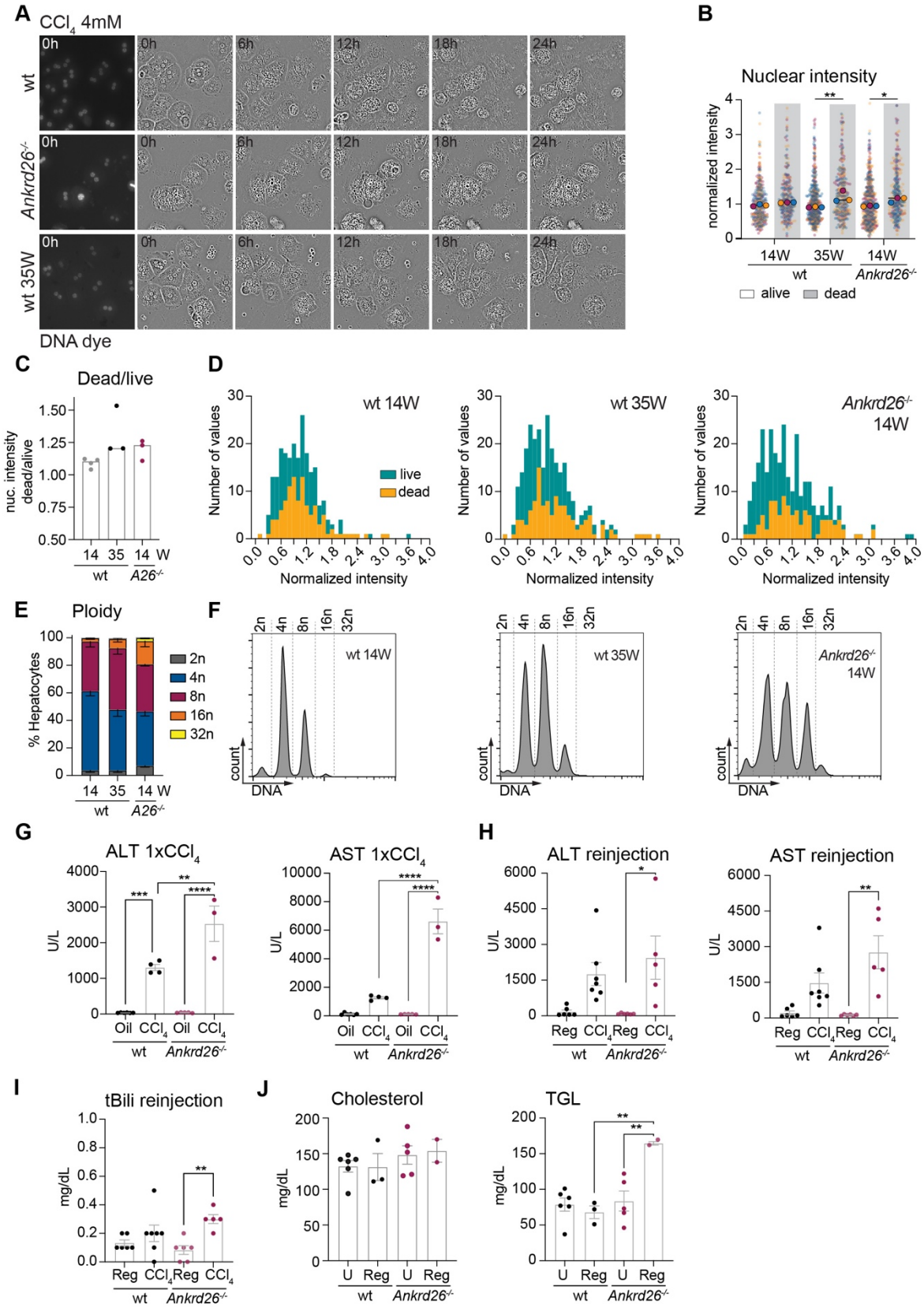
(A) Cyp2E1 transcript levels relative to GAPDH in 10 weeks old wt and *Ankrd26*$^{-/-}$ livers. N=5 mice per genotype. **(B)** Serum concentration of albumin measured for wt and *Ankrd26*$^{-/-}$ mice 3 days after the last injection of 4 weeks chronic CCl_4 or control treatment (Oil). wt oil control N=5, CCl4 N=9; *Ankrd26*$^{-/-}$ oil control N=5, CCl_4 N=10. **(C)** Bodyweight of mice undergoing biweekly CCl_4 (filled circles) or corn oil injections (open circles) as in (B). After 28 days of recovery (N=9), CCl_4-treated mice of either genotype reached a bodyweight comparable to untreated, age-matched mice (yellow filled circles, wt N=3, *Ankrd26*$^{-/-}$ N=5). The dashed line indicates the last injection. **(D)** Serum cholesterol and **(E)** triglyceride (TGL) concentrations as in (B). wt oil control N=5, CCl_4 N=8; *Ankrd26*$^{-/-}$ oil control N=5, CCl_4 N=7. **(F)** Cyp2E1 transcript levels in 10 weeks old *Plk4*$^{f/f}$*; Centrin-GFP*$^{tg/tg}$ (f/f, N=3) and *Plk4*^{LKO}*; Centrin-GFP*$^{tg/tg}$ (LKO, N=4) mice. **(G)** Representative H&E-stained liver sections from *Plk4*$^{f/f}$ and *Plk4*^{LKO} mice following chronic CCl_4 treatment. White dashed lines outline necrotic regions. Scale bar = 100µm **(H)** Necrotic area measured in chronically CCl_4 exposed *Plk4*$^{f/f}$ and *Plk4*^{LKO} livers. N=6 mice per genotype. **(I)** Liver to bodyweight ratio of *Plk4*$^{f/f}$ and *Plk4*^{LKO} mice that were untreated (U) or chronically exposed to CCl_4. *Plk4*$^{f/f}$ U: N=6; CCL_4: N=7; *Plk4*^{LKO} U: N=7; CCL_4: N=7. **(J-M)** Serum concentrations of (J) ALT, (K) AST, (L) total bilirubin, and (M) Albumin. *Plk4*$^{f/f}$ U and CCL_4: N=6; *Plk4*^{LKO} U and CCL_4: N=7. Data for untreated mice in I-M are the same as in Fig.1K-L and shown here for comparison. **(N)** Bodyweight of mice of the indicated genotypes during chronic CCl_4 treatment as in (C). Data are represented as mean ± S.E.M and statistical significance was assessed by an unpaired two-tailed Student's t-test (A, F, H), by one-way-ANOVA with Sidak's multiple comparisons test (B, D, E, I-M), or by multiple unpaired two-tailed t-tests with Sidak-Bonferroni correction for multiplicity (C, N; comparisons were calculated for each genotype between treatment groups). Only significant results are indicated. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$.



Supplemental Figure 5: ANKRD26-mediated ploidy control restricts hepatocyte hyperpolyploidization during chronic liver injury.

(A) Cell density quantified on H&E-stained liver sections of mice chronically exposed to CCl_4 or age matched, untreated (U) controls. *Plk4*$^{f/f}$*; Centrin-GFP*$^{tg/tg}$ (f/f): U N=3, CCl_4 N=7; *Plk4*^{LKO}*; Centrin-GFP*$^{tg/tg}$ (LKO): U N=3, CCl_4 N=6. **(B-C)** Immunohistochemistry staining for Ki67 on paraffin-embedded liver sections was quantified to assess the proliferation status in untreated (U) and regenerated (Reg) livers from the indicated genotypes. Scale bar represents 50 µm. N=3 per

genotype and condition. Yellow arrows indicate Ki67+ hepatocytes, red arrow points out a Ki67+ nonhepatocyte. **(D)** Liver zonation was measured in immunostained sections as described in Fig. S1D. N=3 mice per genotype and treatment. (Reg) n=15-26 wt and n=36-71 *Ankrd26*$^{-/-}$ liver lobules were measured. Data for untreated livers is the same as in Fig. 2J and shown here for comparison. **(E)** Representative examples of regenerated wt and *Ankrd26*$^{-/-}$ livers stained for glutamine synthetase (GS) and E-cadherin. Scale bar = 100µm. **(F)** Liver sections from mice immunostained for ZO-1 to mark cell boundaries and the pan-leukocyte marker CD45. Scale bar represents 10 µm. **(G)** The number of CD45^{+} cells quantified in liver sections as shown in (F). N=3 mice per genotype. **(H)** Mean ploidy was measured by flow cytometry on hepatocytes isolated from wt and *Ankrd26*$^{-/-}$ mice 28 days after regeneration from chronic CCl_4 treatment (Reg) or untreated age-matched controls (U). N=3 mice per genotype. **(I)** Number of mitotic cells per area normalized to cell density in *Plk4*$^{f/f}$*; Centrin-GFP*$^{tg/tg}$ (f/f, N=7) and *Plk4*^{LKO}*; Centrin-GFP*$^{tg/tg}$ (LKO, N=7) livers 3 days after the last injection of a chronic CCl_4 treatment scheme. **(J)** Examples of hepatocytes with aberrantly shaped nuclei or >2 nuclei per cell from *Ankrd26*$^{-/-}$ mice that recovered for 28 days from chronic CCl_4 treatment. White dashed lines indicate cell boundaries. Scale bar represents 10 µm. Data are represented as mean ± S.E.M and Statistical significance was assessed by an unpaired two-tailed Student's t-test (I), one-way-ANOVA (A, B, G, H) or two-way ANOVA (D) both with Sidak's multiple comparisons test. Only significant results are indicated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.


A
CCl4 4mM
wt
Ankrd26-/-
wt 35W
0h
6h
12h
18h
24h
DNA dye
B
Nuclear intensity
normalized intensity
14W
35W
wt
Ankrd26-/-
alive
dead
C
Dead/live
nuc. intensity dead/alive
D
wt 14W
wt 35W
Ankrd26-/- 14W
Number of values
live
dead
Normalized intensity
E
Ploidy
% Hepatocytes
2n
4n
8n
16n
32n
F
count
DNA
G
ALT 1xCCl4
AST 1xCCl4
U/L
Oil
CCl4
H
ALT reinjection
AST reinjection
Reg
I
tBili reinjection
mg/dL
J
Cholesterol
TGL
U

Supplemental Figure 6: Increased ploidy does not sensitize hepatocytes to CCL_4-induced death.

(A) Representative images show live imaging of hepatocytes isolated from 14 or 35 weeks old wt mice and 14 weeks old *Ankrd26*$^{-/-}$ animals treated for 24h with CCl_4 (4 mM) or DMSO (not shown). Nuclear fluorescence intensity (generated from a cell-permeable DNA dye) was measured at 0h. Cell death was defined by the onset of membrane blebbing and detachment. **(B)** Median normalized nuclear intensity of surviving (white) and dead (grey) hepatocytes. Transparent dots represent all cells, filled circles show the median per mouse. Median of all mice is shown as a line. Statistical significance was determined on N=3 mice. For each mouse n=207-292 cells were analyzed. **(C)** Ratios of the median nuclear intensity of dead over surviving cells per mouse of the animals shown in (A). **(D)** Representative histograms of nuclear intensity as a read-out for DNA content showing cells that survived (green) or died (yellow) during 24h of CCl_4 exposure. To correct for differences in dye distribution across the well, the nuclear intensity was normalized to the median intensity per frame. **(E)** Quantification of the ploidy distribution of wt and *Ankrd26*$^{-/-}$ (*A26*$^{-/-}$) hepatocytes at 14 and 35 weeks of age. **(F)** Representative histograms of the DNA distribution measured by flow cytometry of the same mice as shown in (D) and quantified in (E). **(G)** Alanine transaminase (ALT) and aspartate transaminase (AST) serum concentrations from mice of the indicated genotypes analyzed 17h after injection with a single dose of corn oil or 5 ml/kg CCl_4. wt control N=5, CCl_4 N=4; *Ankrd26*$^{-/-}$ control N=5, CCl_4 N=3. **(H)** ALT and AST concentrations measured in the serum of mice regenerated for 28 days from chronic CCl_4 exposure and left untreated (Reg) or re-injected (CCl_4) with a single dose of CCl_4 as in (G). **(I)** Total bilirubin (tBili) serum concentration as in (H). **(J)** Cholesterol and triglyceride (TGL) serum concentrations for untreated (U) or regenerated (Reg) mice as in (H). The untreated animals are the same as in Fig S2E and were included here for comparison. Data in (B-C) are shown as median, data in (E, G-J) are represented as mean ± S.E.M. Statistical significance was assessed using one-way-ANOVA with Sidak's multiple comparisons test (B-C, G-J). Only significant results are indicated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.