Akt-mediated FoxO1 inhibition is required for liver regeneration

Montse Pauta, Noemi Rotllan, Ana Fernández-Hernando, Cedric Langhi, Jordi Ribera, Loreto Boix, Jordi Bruix, Wladimiro Jimenez, Yajaira Suárez, David A. Ford, Angel Baldán, Morris J. Birnbaum, Manuel Morales-Ruiz, Carlos Fernández-Hernando

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Isolation of mouse hepatocytes and cell culture. Freshly isolated primary hepatocytes were obtained from the liver of 12-week-old male C57BL/6J mice (Charles River). Liver capsule was disrupted after collagenase A (Roche Diagnostics, Basel, Switzerland) retrograde perfusion through portal vein and the suspension was filtered through a 100 µm cell strainer. Hepatocytes were purified by centrifugation at 70 *g* for 1 minute, twice. Cells were seeded ($7x10^5$ cells/well) in collagen I six-well culture-slides (BD Pharmigen, San Diego, CA, USA) and cultured at 37°C, 5% CO_2, and 95% relative humidity in Dulbecco's Modified Eagle Medium (DMEM) with 50 U/mL penicillin, 50 mg/mL streptomycin, 10% fetal calf serum and supplemented with 7.5 µg/mL hydrocortisone, 5 mL ITS and 20 ng/mL hepatocyte growth factor (Sigma-Aldrich, St. Louis, MO, USA).

Cell treatments. Following adhesion, cells were washed to remove unattached hepatocytes and subsequently cultured in serum-free medium for 24 hours with 5 µM Akt1/2 kinase inhibitor A6730 (Sigma-Aldrich, St. Louis, MO, USA), 1 µM FoxO1 inhibitor AS1842856 (Merk Millipore, Billerica, MA, USA) or both. After this period of time, we stimulated the cells with 10%FBS maintaining the drug treatments overnight.

TUNEL staining. DNA fragmentation was assessed by TUNE staining using the In situ Cell Death Detection Kit-TMR red (Roche, Mannheim, Germany), according to the manufacturers' instructions. Briefly, frozen liver sections of 8-µm were fixed in 4%

paraformaldehyde, pH7.4 for 20 min, washed with PBS buffer and incubated in permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 minutes on ice. After washing the slides twice with PBS buffer, 50 mL of the TUNEL reaction mixture was added to the slides and the samples were incubated in a humidified atmosphere for 60 min at 37ºC in the dark. After extensive washing with PBS buffer, the slides were evaluated under a fluorescence microscope (Nikon Eclipse E600, Kawasaki, Kanagawa, Japan). The tissue was counterstained with DAPI to visualize the nuclei. The positive control for the TUNEL assay was generated by treating the tissue with DNase I (3000 U/mL) for 10 min at room temperature to induce DNA strand breaks, prior to the labeling procedure.

SUPPLEMENTAL RESULTS

Pharmacological inhibition of FoxO1 up-regulates the expression of positive cell cycle markers in primary hepatocytes.

To further investigate the functional impact of the Akt/FoxO1 pathway in the activation of the cell cycle, we treated primary hepatocytes isolated from wild-type mice with Akt1/2 and FoxO1 inhibitors. As we wanted to accurately reproduce the Akt1/Akt2/FoxO1 deficiency generated in the TLKO mice, we selected the A6730 (Akt1/2 inhibitor) and the AS1842856 (FoxO1) inhibitors on the basis of their specificity for their targets. For instance, A6730 has been shown to specifically inhibit Akt phosphorylation without affecting the activity of other closely related AGC family kinases[1], such as PKA or PKC. Concerning the AS1842856 inhibitor, this inhibitor preferentially inhibits the activity of FoxO1 over that of the related Foxo3a and Foxo4 proteins (70%, 20%, and 3% inhibition tested in the HepG2 cell line, respectively)[2]. Primary hepatocytes were incubated in serum free conditions for 24 hours in the presence or absence of the inhibitors. After this period of time, we stimulated the cells with 10%FBS overnight maintaining the drug treatments and next we assessed the protein expression of PCNA, CCND1 and Ki-67 by western blot. As shown in the supplemental figure S3,

the single inhibition of FoxO1 (AS1842856 treatment) or the simultaneous inhibition of Akt and FoxO1 (combined A6730 and AS1842856 treatment) resulted in the overexpression of PCNA and CCND1, compared with non-treated or A6730 treated hepatocytes. We also observed the overexpression of Ki-67 in cells treated with AS1842856, compared with the rest of experimental conditions and a modest increase of this cell cycle marker in hepatocytes treated simultaneously with Akt and FoxO1 inhibitors, compared with the single A6730 treatment. Therefore, FoxO1 inhibition in hepatocytes, results in the overexpression of positive cell cycle regulators.

SUPPLEMENTAL REFERENCES

1. Barnett SF, Defeo-Jones D, Fu S, Hancock PJ, Haskell KM, Jones RE, et al. Identification and characterization of pleckstrin-homology-domain-dependent and isoenzyme-specific Akt inhibitors. Biochem J 2005; 385: 399-408.

2. Nagashima T, Shigematsu N, Maruki R, Urano Y, Tanaka H, Shimaya A, et al. Discovery of Novel Forkhead Box O1 inhibitors for treating type 2 diabetes: Improvement of fasting glycemia i diabetic db/db Mice. Molecular Pharmacology 2010; 78: 961-970.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Akt deficiency does not influence glycogen content. (**D**) Glycogen content analysis in livers from WT, DLKO and TLKO (n=5 for each experimental group) assessed by periodic acid-Schiff (PAS) staining. Pink color indicates the presence of hepatic glycogen before (0 days) and after PH (2, 4 and 6 days).

Figure S2. Genetic ablation of hepatic AKt1/Akt2 or Akt1/Akt2/FoxO1 does not influence CCND1 expression or cell death in resting livers. (**A**) Representative immunostaining for the CCND1 antigen (left panels) in liver of WT, DLKO and TLKO mice before PH. Merged images on right panels show co-localization of CCND1 (red) and nuclear DNA (DAPI, blue). Liver from WT

mice after four days post-PH was used as a positive control for the CCND1 immunohistochemistry (bottom panels, ct+).). Original magnification 100x, (n=5). (**B**) Representative TUNEL staining (left panels, TMR-dUTP) in liver of WT, DLKO and TLKO mice before PH. Merged images on right panels show co-localization of TMR-dUTP (red) and nuclear DNA (DAPI, blue). Liver from WT mice before PH was treated with DNase I to induce DNA strand breaks prior to the labeling procedure and used as a positive control for TUNEL assay (bottom panels, ct+). Original magnification 100x, (n=5).

Figure S3. Pharmacological inhibition of FoxO1 up-regulates the expression of proliferative and positive cell cycle markers in mouse primary hepatocytes. (**A**), Mouse primary hepatocytes isolated from WT mice were treated with or without the Akt1/2 inhibitor A6730 (5 mM) and the FoxO1 inhibitor AS1842856 (1 mM), as indicated in the figure. Lysates (60 mg of proteins) were analyzed by Western blotting with specific antibodies for the indicated proteins, (n=3). Tubulin was used as loading control. Bottom panels show the densitometry analysis of CCND1, PCNA and Ki-67 expression in the presence or in the absence of the A6730 and AS1842856 treatments. Results are represented as mean ± S.E.M. *$P<0.05$ vs. non-treated and A6730 treated cells, $^{\#}P<0.05$ vs. all the other experimental conditions, $^{\&}P<0.05$ vs. A6730 treated cells. (**C**), Representative phase-contrast images of mouse primary hepatocytes treated or not with A6730 and AS1842856 inhibitors.

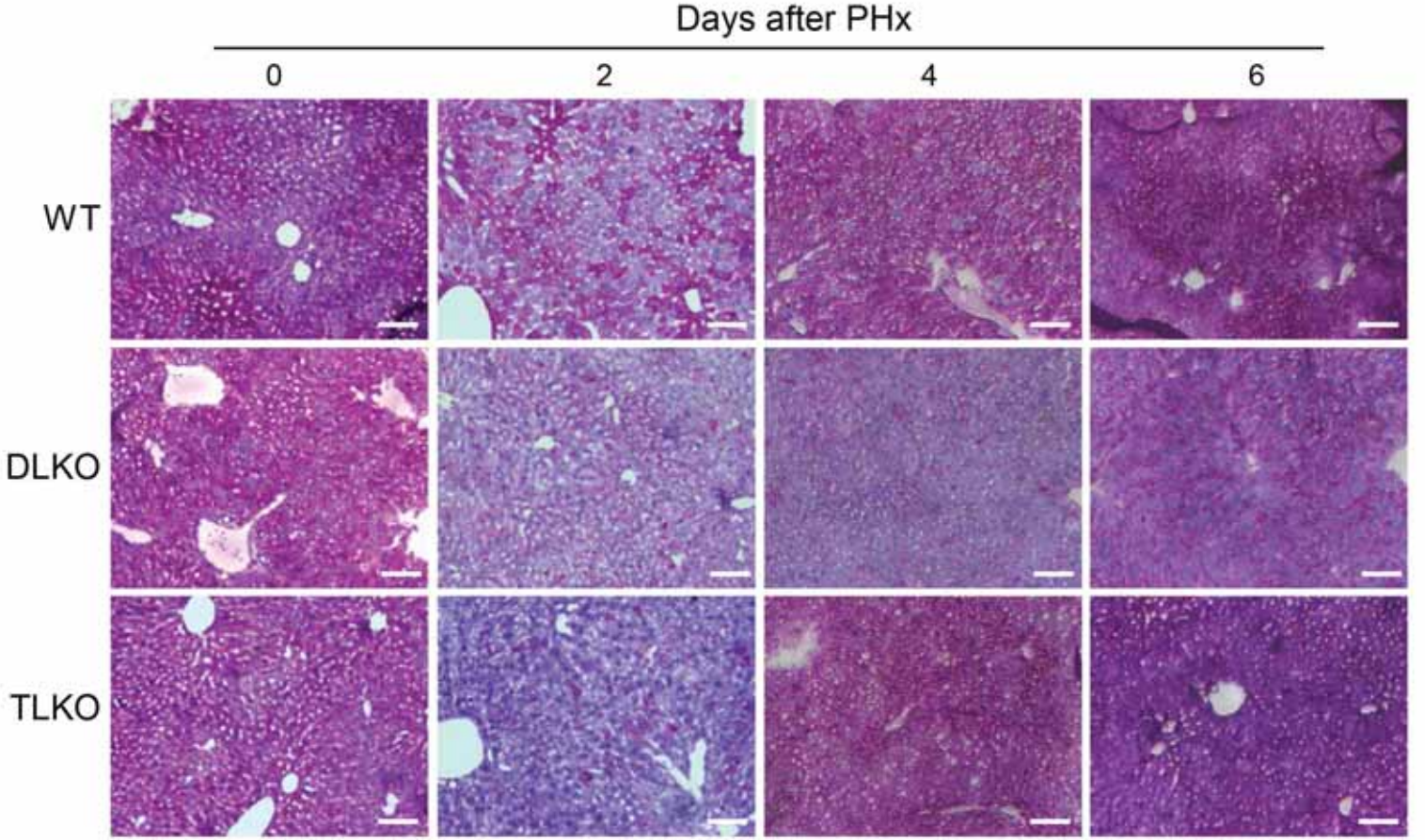


Figure S1

Figure S2

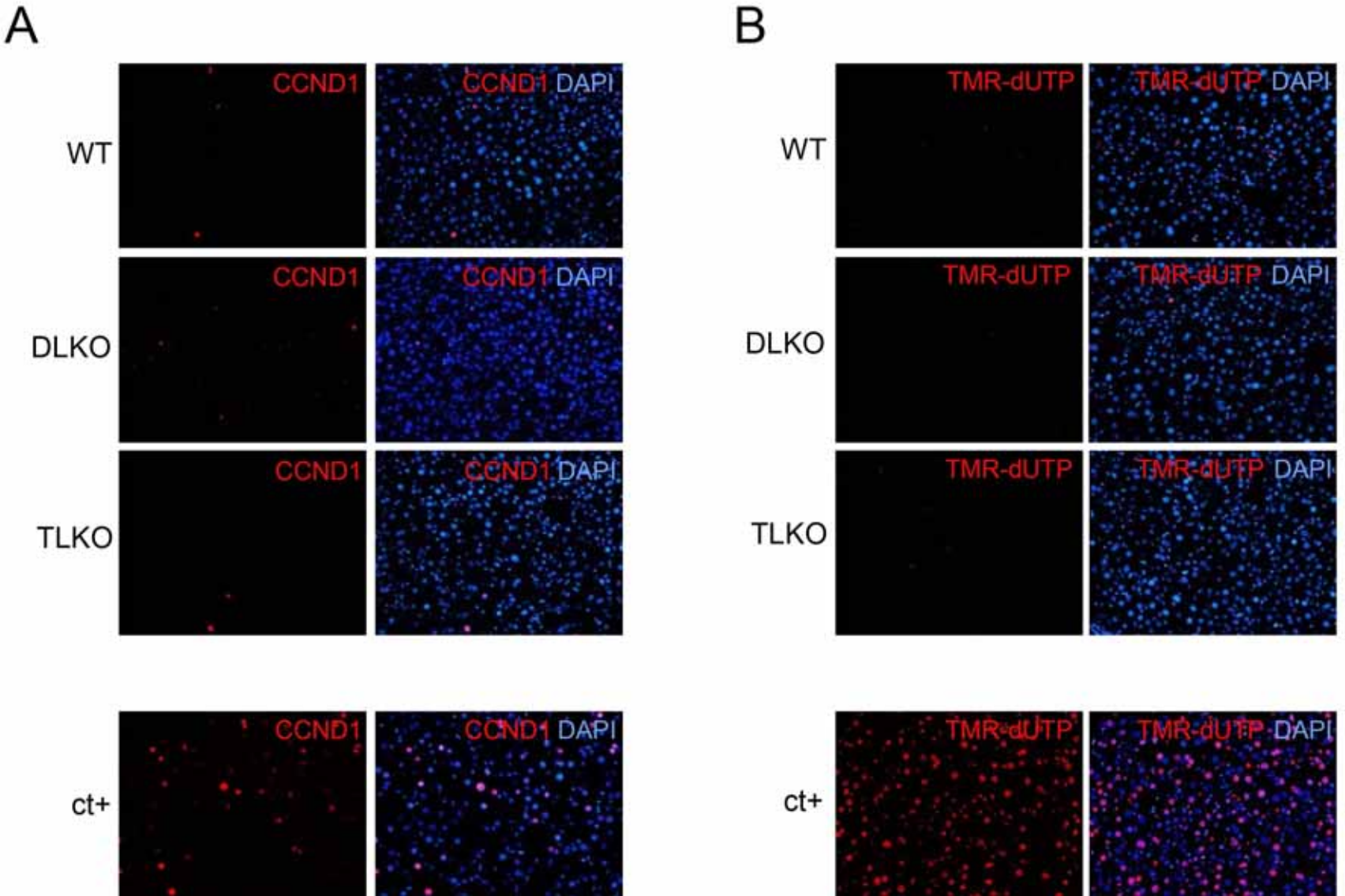
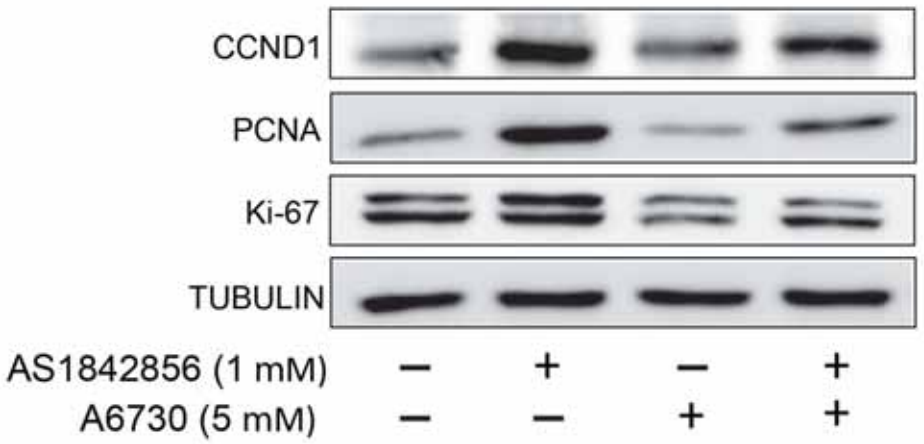
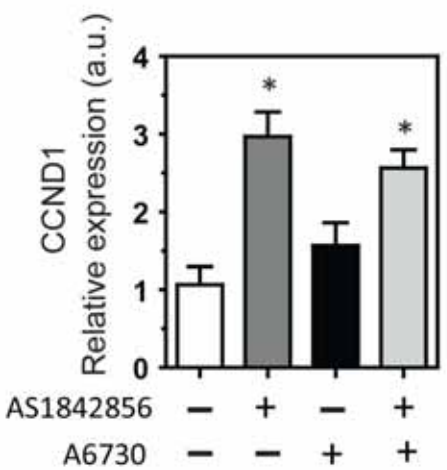
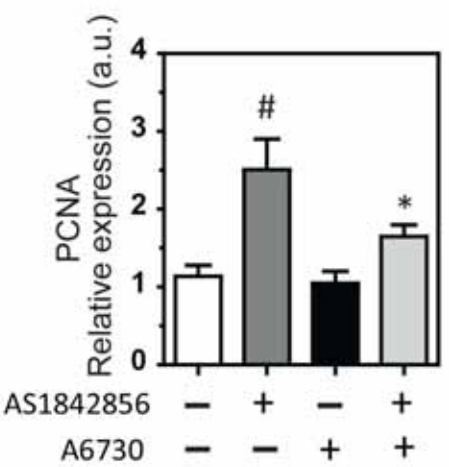

A
CCND1
CCND1 DAPI
WT
DLKO
TLKO
ct+
B
TMR-dUTP
TMR-dUTP DAPI
WT
DLKO
TLKO
ct+

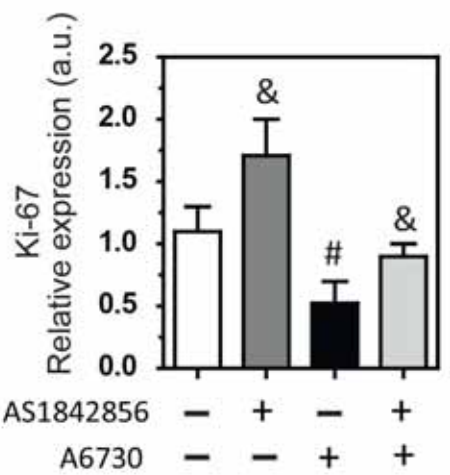
Figure S3

A



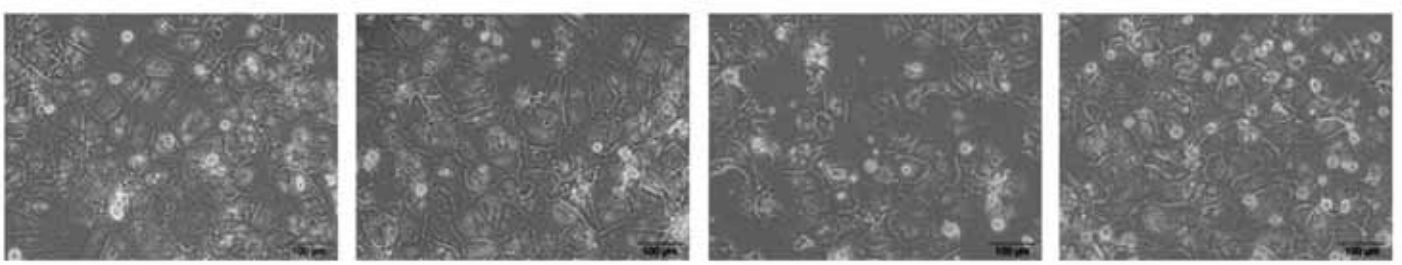






B

Freshly isolated primary hepatocytes



Vehicle | AS1842856 (1mM) | A6730 (5mM) | AS1842856 (1mM) A6730 (5mM)