Title

NRF2 activates growth factor genes and downstream AKT signaling to induce mouse and human hepatomegaly

Journal

Journal of Hepatology, 72(6)

ISSN

0168-8278

Authors

He, Feng
Antonucci, Laura
Yamachika, Shinichiro
et al.

Publication Date

2020-06-01

DOI

10.1016/j.jhep.2020.01.023

Peer reviewed

HHS Public Access

Author manuscript

J Hepatol. Author manuscript; available in PMC 2021 April 19.

Published in final edited form as:

J Hepatol. 2020 June ; 72(6): 1182–1195. doi:10.1016/j.jhep.2020.01.023.

NRF2 activates growth factor genes and downstream AKT signaling to induce mouse and human hepatomegaly

Feng He[#1], **Laura Antonucci**[#1], **Shinichiro Yamachika**[#1], **Zechuan Zhang**[3], **Koji Taniguchi**[1], **Atsushi Umemura**[1], **Georgia Hatzivassiliou**[4], **Merone Roose-Girma**[4], **Miguel Reina-Campos**[5], **Angeles Duran**[5], **Maria T. Diaz-Meco**[5], **Jorge Moscat**[5], **Beicheng Sun**[3,*], **Michael Karin**[1,2,*]

[1]Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA;

[2]Department of Pathology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA;

[3]Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing 210008, Jiangsu Province, China;

[4]Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA;

[5]Cancer Metabolism and Signaling Networks Program, Sanford-Burnham-Prebys Medical Discovery Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

[#] These authors contributed equally to this work.

Abstract

Background & Aims: Hepatomegaly can be triggered by insulin and insulin-unrelated etiologies. Insulin acts via AKT, but how other challenges cause hepatomegaly is unknown.

Methods: Since many hepatomegaly-inducing toxicants and stressors activate NRF2, we examined the effect of NRF2 activation on liver size and metabolism using a conditional allele encoding a constitutively active NRF2 variant to generate $Nrf2^{Act\text{-}hep}$ mice in which NRF2 is selectively activated in hepatocytes. We also used adenoviruses encoding variants of the autophagy adaptor p62/SQSTM1, which activates liver NRF2, as well as liver-specific ATG7-deficient mice

[*]Corresponding authors. Addresses: Laboratory of Gene Regulation and Signal Transduction, Departments of Pharmacology and Pathology, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA. Tel.: +1 858 534 1361; fax: +1 858 534 0848 (M. Karin), or Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing 210008, Jiangsu Province, China. Tel.: +86 25 83105892; fax: +86 25 86560946 (B. Sun). karinoffice@ucsd.edu (M. Karin), sunbc@nju.edu.cn (B. Sun).

Authors' contributions

F.H., L.A., and S.Y. designed and performed the main experiments and wrote the paper with M.K., who conceived and supervised the project. K.T., A.U., G.H. and M.G. participated in the main experiments. G.H. and M.G. provided mutant NRF2 mice used by L.A. to generate $Nrf2^{Act}$ mice. F.H. and S.Y. generated p62 adenovirus. Z.Z. and B.S. provided and analyzed human samples. M.R.C., A.M., M.T.D.-M. and J.M. performed the RNA-seq analyses. All authors discussed and interpreted the results and revised the manuscript.

Conflict of interest

G.H and M.G. are full time employees of Genentech/Roche and hold company shares. Remaining authors declare no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Supplementary data

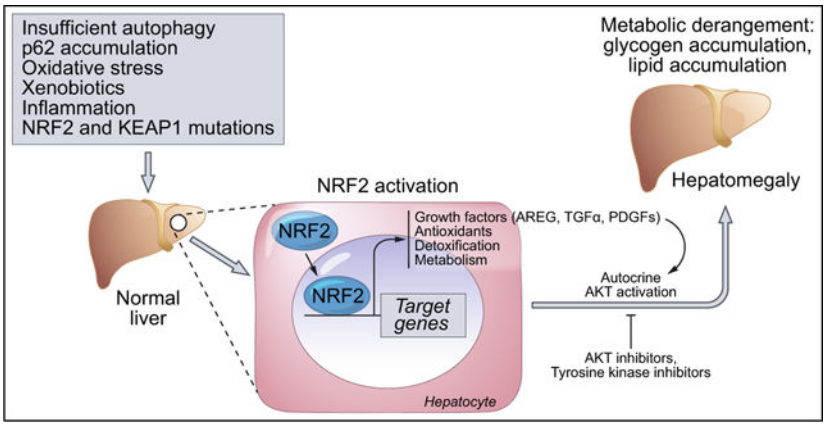
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2020.01.023.

(*Atg7*$^{\Delta hep}$) and liver specimens from patients with hepatic sinusoidal obstruction syndrome (HSOS) and autoimmune hepatitis (AIH). RNA sequencing and cell signaling analyses were used to determine cellular consequences of NRF2 activation and diverse histological analyses were used to study effects of the different manipulations on liver and systemic pathophysiology.

Results: Hepatocyte-specific NRF2 activation, due to p62 accumulation or inhibition of KEAP1 binding, led to hepatomegaly associated with enhanced glycogenosis, steatosis and G2/M cell cycle arrest, fostering hyperplasia without cell division. Surprisingly, all manipulations that led to NRF2 activation also activated AKT, whose inhibition blocked NRF2-induced hepatomegaly and glycogenosis, but not NRF2-dependent antioxidant gene induction. AKT activation was linked to NRF2-mediated transcriptional induction of PDGF and EGF receptor ligands that signaled through their cognate receptors in an autocrine manner. Insulin and insulin-like growth factors were not involved. The NRF2-AKT signaling axis was also activated in human HSOS- and AIH-related hepatomegaly.

Conclusions: NRF2, a transcription factor readily activated by xenobiotics, oxidative stress and autophagy disruptors, may be a common mediator of hepatomegaly; its effects on hepatic metabolism can be reversed by AKT/tyrosine kinase inhibitors.

Graphical Abstract



Lay summary

Hepatomegaly can be triggered by numerous etiological factors, including infections, liver cancer, metabolic disturbances, toxicant exposure, as well as alcohol abuse or drug-induced hepatitis. This study identified the oxidative stress response transcription factor NRF2 as a common mediator of hepatomegaly. NRF2 activation results in elevated expression of several growth factors. These growth factors are made by hepatocytes and activate their receptors in an autocrine fashion to stimulate the accumulation of glycogen and lipids that lead to hepatocyte and liver enlargement. The protein kinase AKT plays a key role in this process and its inhibition leads to reversal of hepatomegaly.

Keywords

p62/SQSTM1; Autophagy; NRF2; HSOS; Hepatomegaly; AKT; PDGFR; EGFR

Introduction

Hepatomegaly, or liver enlargement, is a non-specific patho-logical reaction triggered by infections, liver cancer, metabolic disturbances, toxicant exposure, as well as alcohol abuse or drug-induced hepatitis.[1] Congenital disorders, including hemolytic anemia, polycystic liver disease, sickle cell disease, hereditary fructose intolerance or carnitine palmitoyltransferase deficiency can also elicit hepatomegaly.[2–6] Hepatomegaly has been frequently observed in patients with type I and type II diabetes with poor glycemic control.[7,8] In such cases, hepatomegaly has been attributed to hepatocyte glycogen accumulation driven by insulin-induced hepatic glycogenosis, especially in pediatric patients.[8] By contrast, the mechanisms underlying hepatomegaly caused by other etiologies are poorly understood. Histopatho-logical analysis suggests the involvement of vascular swelling and inflammation, as well as increased accumulation of iron, glycogen, fat or insoluble proteins within hepatocytes.[1] Mimicking insulin overdosing, adenovirus-mediated hepatic overexpression of a constitutively active form of AKT, the key effector of insulin signaling,[9] resulted in pronounced hepatomegaly accompanied by hypoglycemia and hypertriglyceridemia.[10]

Autophagy defects can also induce hepatomegaly. Indeed, both liver-specific *Atg5* (*Atg5*$^{\Delta hep}$) and *Atg7* (*Atg7*$^{\Delta hep}$) knockout mice exhibit marked liver enlargement.[11–16] Autophagy-deficient livers show accumulation of the autophagy substrate and signaling scaffold p62/SQSTM1 (hereafter referred to p62), which sequesters Kelch-like ECH-associated protein 1 (KEAP1) away from the oxidant-responsive transcription factor nuclear factor erythroid 2-related factor 2 (NRF2), resulting in nuclear accumulation and activation of NRF2.[13,16,17] Nuclear NRF2 also accumulates in response to oxidative stress and KEAP1 inactivation.[18,19] Normally, NRF2 plays a critical role in the induction of genes that code for cytoprotective redox-active proteins and enzymes that detoxify reactive oxygen species (ROS) and xenobiotics.[18] But NRF2 can also undergo persistent activation in lung, liver and colon cancers due to gain-of-function mutations in the *NFE2L2* gene that prevent binding to KEAP1 or loss-of-function mutations in the *KEAP1* gene.[20–23] In addition to NRF2, several other factors may contribute to hepatomegaly in the autophagy-deficient liver, including YAP,[24] mTORC1,[11] and FXR.[25] Thus, it is not clear whether NRF2 activation alone is sufficient for induction of hepatomegaly in oxidatively stressed or autophagy-deficient livers. Also, the relationship between the NRF2-induced protective response and hepatomegaly remains unknown.

NRF2-deficient mice are susceptible to numerous electro-philes and oxidants,[18,26] and also exhibit defective liver regeneration due to oxidative stress-mediated insulin/insulin-like growth factor (IGF) resistance.[27] Conversely, NRF2 activation redirects glucose and glutamine into the anabolic pentose phosphate pathway when superimposed on persistently activated AKT in cancer cells that exhibit chronic upregulation of phosphatidylinositol 3-kinase (PI3K) signaling.[28] Although in cancer cells NRF2 activation confers survival and growth advantage,[29–32] in autophagy-defective livers NRF2 activation was reported to provoke hepatocyte death and liver injury.[12,13,16,25,33] How NRF2 activation promotes liver injury in the absence of autophagy is unknown. Moreover, persistent activation of NRF2 in an autophagy competent liver does not cause liver injury as shown by liver-specific *Keap1*

knockout (*Keap1*$^{\Delta hep}$) mice, which exhibit hepatomegaly without liver damage.[12] Evidently, the effects of persistent NRF2 activation are highly context dependent.

We previously found that in the unstressed liver, p62 controls basal NRF2 activity[32] and that p62 accumulation due to autophagy disruption contributes to liver and pancreatic tumorigenesis by activating NRF2.[30,32,34] To further understand the role of persistent NRF2 activation in hepatocytes and whether it is sufficient to induce hepatomegaly, we generated transgenic mice that express a KEAP1-resistant form of NRF2 in their hepatocytes (*Nrf2*$^{Act\text{-}hep}$). We also used p62 adenovirus vectors to selectively overexpress p62 in hepatocytes. Here, we demonstrate that hepatocyte-specific activation of NRF2 by either method causes marked hepatomegaly, glycogenosis, hypoglycemia and hypertriglyceridemia. Unexpectedly, we found that persistent NRF2 activation leads to hepatomegaly via upregulation of AKT signaling and that the latter depends on autocrine epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) signaling. The NRF2-AKT axis is also activated in human hepatomegaly.

Materials and methods

Mouse experimentation

Nfe2l2$^{iE79Q/+}$ mice were generated at Genentech using C57BL/6N embryonic stem cells and standard methodology. A targeting cassette containing the *loxP* sequence, a duplicated copy of the last 130 bp of *Nfe2l2* intron 1, wild-type (WT) *Nfe2l2* cDNA (exons 2–5), a human growth hormone 3' UTR followed by a 4× poly-adenylation signal, an *FRT-Neo-FRT* selection marker and a second *loxP* sequence was inserted into the *Nfe2l2* locus 130 bp 5' to a mutated exon 2 encoding the E79Q (GAA to CAA) mutation. The *Neo* cassette was excised in embryonic stem cells using FLP recombinase prior to electroporation. All alleles were maintained on a C57BL/6N genetic background. Upon Cre action, the floxed cassette is excised leaving a single *loxP* site, and exon 1 now splices to the modified exon 2 instead of the WT *Nfe2l2* cDNA. *Sqstm1/p62*$^{F/F}$, *Atg7*$^{F/F}$, *Raptor*$^{F/F}$ and *Ikkβ*$^{F/F}$ mice[15,35–37] were intercrossed with Alb-Cre mice (C57BL/6, Jackson Lab) to generate *Sqstm1/p62*$^{F/F}$*;Alb-Cre* (*p62*$^{\Delta hep}$), *Atg7*$^{F/F}$*-Alb-Cre* (*Atg7*$^{\Delta hep}$), *Raptor*$^{\Delta hep}$ and *Ikk*b$^{\Delta hep}$ mice. B6.129X1-Nfe2l2tm1Ywk/J (*Nrf2*$^{-/-}$) mice were previously described[38] and back-crossed into the C57BL/6 background. *Atg7*$^{\Delta hep}$ and *p62*$^{\Delta hep}$ mice were crossed to generate *Atg7*$^{\Delta hep}$*; p62*$^{\Delta hep}$ mice. All mice were maintained in filter-topped cages on autoclaved food and water and experiments were performed according to UCSD Institutional Animal Care and Use Committee and NIH guidelines and regulations. Dr. Karin's Animal Protocol S00218 was approved by the UCSD Institutional Animal Care and Use Committee. See the Supplementary Information for further details.

Human hepatomegaly clinical samples

Data were obtained from liver tissues of 5 male and 5 female patients with HSOS-related hepatomegaly and 4 male and 6 female control livers (patients with hepatic hemangioma), as well as 3 male and 2 female patients with AIH-related hepatomegaly, and 3 male and 2 female control livers (patients with hepatic hemangioma). Liver tissues were acquired between June 2018 and June 2019 at The Affiliated Drum Tower Hospital of Nanjing

University Medical School (Nanjing, Jiangsu, China). Patients with hepatomegaly ranged from 27 to 54 years old, with a mean age of 40.7 years. Eight patients with HSOS suffered from ascites and 9 patients suffered from jaundice. Patients with hepatic hemangioma ranged from 29 to 62 years, with a mean age of 44.6 years. Control individuals with normal liver histology had normal levels of aminotransferases with no evidence of ascites, jaundice or hepatomegaly. The study was approved by the Institutional Ethics Committee of The Affiliated Drum Tower Hospital with IRB #2018-289-01. Informed consent for tissue analysis was obtained before surgery. Detailed characteristics of hepatomegaly in patients with HSOS and AIH as well as controls are listed in Tables S1 and S2. All research was performed in compliance with government policies and the Helsinki declaration. All experiments were undertaken with the understanding and written consent of each participant.

RNA-seq analysis and data processing

Single end 50 bp reads were obtained by RNA sequencing (RNA-seq). FASTQC module was run on FASTq files to check data quality. Quality scores for raw reads were Sanger transformed using FASTq Groomer. FASTq Groomer outputs were aligned to mm10 genome using TopHat (-first strand) in local sensitive model. Aligned reads were sorted by coordinates using Sort BAM module. Gene expression estimates were calculated using Cufflinks using reference mm10 GTF file from iGenomes. Differential gene expression was calculated for all pairs using CuffDiff module. For gene set enrichment analysis (GSEA), the gene expression matrix was pooled from gene expression estimates from Cufflinks output and processed with human-translated gene symbols with 1,000 permutations using a *t* test metric for gene ranking. Enrichment was tested using default v5.2 MSigDb gene sets. Raw data has been deposited in the NCBI Gene Expression Omnibus (GSE-144865).

Quantification and statistical analysis

Data are shown as mean ± SD as indicated. Statistical significance was determined using 2-tailed Student's *t* test and *p* values lower than 0.05 were considered statistically significant (*p <0.05, **p <0.01; ***p <0.001). GraphPad Prism was used for statistical analysis and graphing.

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

p62 induces NRF2-dependent hepatomegaly

Overexpression of p62 in mouse liver causes hepatocellular carcinoma.[32] To investigate early effects of p62 overexpression that may shed new light on its tumorigenic activity, we used adenovirus (Adv) to transiently express GFP, p62 WT or a p62 variant, p62KIR^{-} (that no longer binds KEAP1), in mouse livers.[13] Transduction of p62 WT, but not p62KIR^{-} or GFP, led to pronounced hepatomegaly, resulting in a 2.5-fold increase in liver to body weight ratio within 7 days of Adv infection (Fig.1A,B).

Histological analysis confirmed numerous p62-expressing hepatocytes in both p62 WT- and p62KIR^{-}-transduced mice (Fig. 1C). Even Adv-GFP infection led to a small increase in endogenous p62 expression, which may reflect Adv-induced inflammation and NF-κB activation.[39] Transduction of p62 WT, but not p62KIR^{-} Adv, induced the NRF2 target genes NAD(P)H quinone dehydrogenase 1 (*Nqo1*) and glutathione S-transferase Mu 1 (*Gstm1*) (Fig. 1D). To determine whether p62-induced hepatomegaly was NRF2-dependent and to examine the role of endogenous p62, with which exogenous p62 may oligomerize, as well as mTORC1 and IKKβ/NF-κB signaling, which are also stimulated by p62,[17] we transduced different mouse mutants with Adv-p62. Only $Nrf2^{-/-}$ mice were resistant to induction of hepatomegaly, whereas Adv-p62 transduction of $p62^{\Delta hep}$, $Raptor^{\Delta hep}$ and $Ikkb^{\Delta hep}$ mice led to as much hepatomegaly as in WT mice (Fig. S1A). These results confirm that NRF2, but neither mTORC1 nor NF-κB, is needed for p62-induced hepatomegaly.

To determine whether NRF2 activation is sufficient for induction of hepatomegaly, we used $Nfe2l2^{iE79Q/+}$ mice that conditionally express an NRF2(E79Q) variant that no longer binds KEAP1 (Fig. S1B), originally identified in lung cancer.[20] We intercrossed these mice with *Alb-Cre* mice to generate homozygous $Nrf2^{Act\text{-}hep}$ mice in which NRF2 is activated in liver parenchymal cells (Fig. 1E). As seen with Adv-p62 overexpression, liver-specific NRF2 activation caused hepatomegaly, which was apparent at 4 weeks of age and maximal at 8 weeks (Fig. 1F), similar to what was seen in the $Keap1^{\Delta hep}$ mice.[12] Histological examination revealed cytoplasmic clearing in 8- and 16-week-old $Nrf2^{Act\text{-}hep}$ mice (Fig. S1C), similar to what was observed in human hepatomegaly caused by insulin overdosing.[8] Of note, no obvious liver injury was observed in the $Nrf2^{Act\text{-}hep}$ mice as evidenced by serum alanine aminotransferase levels (Fig. S1D). As expected, liver-specific NRF2 activation induced NRF2 target genes and proteins (Fig. S1E,F).

NRF2 activation causes liver hypertrophy and G2/M arrest

Tissue enlargement can be due to increased cell number (hyperplasia) or cell size (hypertrophy). To determine the basis for NRF2-induced hepatomegaly, we measured the number of nuclei per high magnification field (HMF) and determined hepatocyte DNA content. While p62 WT transduction decreased the number of nuclei per HMF, it increased the amount of DNA per hepatocyte (Fig. 2A,B). The numbers of Ki67^{+} hepatocytes were not significantly different between GFP-, p62 WT- and p62KIR^{-}-transduced animals (Fig. 2C). Consistent with increased DNA content per hepatocyte, qPCR analysis revealed increased expression of G2/M checkpoint-related genes, including aurora kinase a *(Aurka)*, forkhead box m1 (*Foxm1*), cell division cycle 25c (*Cdc25c*) and cyclin b2 (*Ccnb2*) in p62 WT-transduced livers (Fig. 2D). Increased expression of the G2/M marker transcripts *Aurka, Foxm1*, *Ccnb2*, *Cdc25c* and *Cdc25b* was also seen in $Nrf2^{Act\text{-}hep}$ mice (Fig. 2E). Consistent with these results, hepatocyte-specific NRF2 activation decreased the number of nuclei per HMF, but had no significant effect on hepatocyte DNA content (Fig. S2A) or the number of Ki67^{+} hepatocytes (Fig. S2B). Because p21 plays an important role in regulating hepatocyte proliferation *in vivo* and can prevent cell cycle progression upon activation of organ size homeostatic mechanisms,[40] we examined *Cdkn1a*/p21 expression. NRF2 activation induced *Cdkn1a*/p21 mRNA and protein in mouse liver (Fig. S2C,D). In agreement with a previous finding in pancreatic cancer,[30] *Mdm2* mRNA and protein were upregulated, resulting in

lower p53 protein amounts (Fig. S2C,D). These observations suggest a p53-independent mechanism of *Cdkn1a*/p21 modulation.

To better understand p62-induced changes in the hepatocyte transcriptome, we performed transcriptomic profiling by RNA-seq on total liver RNA extracted from GFP-, p62 WT- and p62KIR^{-}-transduced mice. GSEA revealed that Adv-p62 WT strongly induced gene signatures corresponding to the ROS pathway, G2M checkpoint, fatty acid metabolism and xenobiotic metabolism (Fig. 2F and Fig. S2E). In agreement with our hypothesis that NRF2 is the key downstream target for p62, GSEA of RNA-seq data from *Nrf2*$^{Act\text{-}hep}$ and WT mouse livers showed upregulation of many of the gene sets found to be upregulated in response to p62 overexpression (Fig. 2G and Fig. S2F). In particular, hepatocyte-specific NRF2 activation upregulated genes related to protein secretion and the unfolded protein response, which is known to be activated by NRF2 along with the ROS pathway.[41] NRF2 activation also led to upregulation of gene sets involved in fatty acid metabolism and xenobiotic metabolism, both of which were activated by p62.

NRF2 activation alters glucose and lipid metabolism

Given the phenotypic similarity between p62- or NRF2-induced mouse hepatomegaly and insulin-induced hepatomegaly in humans,[8] we investigated whether p62 overexpression and NRF2 activation affect glucose and lipid metabolism. Strikingly, p62 WT overexpression and hepatocyte-specific NRF2 activation resulted in marked hypoglycemia (Fig. 3A), phenocopying insulin overdosing. However, circulating insulin in these mice was not elevated and was a bit reduced in mice with liver-specific NRF2 activation (Fig. 3B). Nonetheless, similar to insulin overdosing, p62 overexpression and hepatocyte-specific NRF2 activation stimulated accumulation of liver glycogen (Fig. 3C and Fig. S3A), and liver and serum triglycerides (Fig. 3D and Fig. S3B), along with elevated serum cholesterol (Fig. S3C). Furthermore, both hepatic NADPH and the NADPH to NADP ratio was elevated in the livers of p62 overexpression and *Nrf2*$^{Act\text{-}hep}$ mice (Fig. 3E and Fig. S3D).

Consistent with these findings, Adv-p62 induced hepatic expression of genes involved in glycogen, glucose and pentose phosphate metabolism and *de novo* lipogenesis, including glycogen branching enzyme 1 (GBE1), glucose 6 phosphate dehydrogenase (G6PD), hexokinase 2 (HK2), glucokinase (GCK), phosphoribosyl pyrophosphate amidotransferase (PPAT), methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), transaldolase 1 (TALDO1), transketolase (TKT), malic enzyme (ME1), fatty acid synthase (FASN), acetyl-CoA carboxylase 1 (ACC1) and stearoyl-CoA desaturase 1 (SCD1) and Sterol regulatory element-binding protein 1c (SREBP1c) (Fig. 3F). By contrast, expression of the phosphoenolpyruvate carboxykinase 1 (PCK1) gene was suppressed. Genes encoding transporters involved in lipid and sterol uptake, such as CD36, ABCG5 and ABCG8, were also induced, but the LDL receptor gene was suppressed (Fig. 3F).

NRF2 activation also induced expression of genes coding for GBE1, G6PD, TKT, ME1, SREPB1c and ACC1 as well as solute carrier family 7 member 11 (SLC7A11), CD36, ABCG8 and ABCG5 (Fig. S3E). p62 overexpression also led to induction of the glutamate-cysteine ligase catalytic subunit (GCLC) and SLC7A11 (Fig. S3F), both of which maintain redox homeostasis.

NRF2-induced hepatomegaly requires AKT activation

Given the similarity between the effects of p62 and NRF2 on liver metabolism and size and those of insulin, we investigated whether p62 and NRF2 affected some aspects of insulin signaling after all. Remarkably, both p62 and NRF2 strongly induced phosphorylation of AKT, the major insulin signaling effector, at threonine (T) 308 and serine (S) 473, indicative of its activation (Fig. 4A,B). S241 phosphorylation of PDK1, which mediates AKT T308 phosphorylation,[42] was also increased on NRF2 activation and Adv-p62 transduction, which also stimulated p70S6K T389 phosphorylation. Similarly, p62 and NRF2 modestly stimulated glycogen synthase kinase (GSK) 3α/β phosphorylation at S21 (GSK3α) and S9 (GSK3β) and forkhead box O1 (FOXO1) phosphorylation at S256. Curiously, Adv-p62 infection or NRF2 activation downregulated PDGF receptor α (PDGFRα) and EGF receptor (EGFR) expression, although the amounts of the tyrosine phosphorylated receptors barely declined (Fig. 4A–C). Of note, Adv-p62 infection or NRF2 activation did not stimulate phosphorylation of IRS1 at Y989 or Y1229, or phosphorylation of IGF1Rβ at Y1131 (Fig. 4A,B). As expected, Adv-p62 WT, but not p62KIR^{-}, induced upregulation of its target NQO1 and the inactivity of p62KIR^{-} was not due to its under-expression (Fig. 4A). NRF2 activation led to a small decrease in expression of *Irs1* mRNA but hardly affected *Irs2* and *Insr* mRNAs (Fig. S4A). Consistent with its effect on AKT, NRF2 activation attenuated FOXO1 nuclear localization in fasted mice (Fig. S4B). Even though NRF2 activation enhanced p70S6K T389 phosphorylation, it did not affect ribosomal S6 protein or 4E-BP1 phosphorylation and did not alter Myc or cyclin D1 expression (Fig. S4C), suggesting that NRF2 activation does not modulate these aspects of translational control.

We examined whether AKT activation was responsible for p62-induced hepatomegaly by treating Adv-p62 infected mice with the AKT inhibitor (AKTi) MK2206 (Fig. S4D). AKTi treatment blocked, as expected, both T308 and S473 phosphorylation (Fig. S4E), and completely prevented p62-induced hepatomegaly (Fig. 4D). AKTi treatment modestly attenuated the decrease in PDGFRα expression and strongly decreased GSK3α/β phosphorylation, without affecting EGFR tyrosine phosphorylation (Fig. S4E). AKTi treatment also blocked the Adv-p62 elicited induction of G2/M marker transcripts (Fig. 4E), reduced the expression of Adv-p62-induced *G6pd* and *Mthfd2* mRNAs and reversed the decrease in *Pdk2* and *Pck1* mRNAs, without affecting the classic NRF2 target genes *Nqo1* and *Gclc* (Fig. 4F). Beyond hepatomegaly, AKTi treatment inhibited Adv-p62-induced liver glycogen accumulation (Fig. S4F). Primary *Nrf2*$^{Act\text{-}hep}$ hepatocytes treated with AKTi showed reduced AKT S473, IRS1 Y1229 and Y989 and p70S6K T389 phosphorylation and less of a decrease in EGFR expression, without an effect on NQO1 and PDK1 expression (Fig. S4G). AKTi treatment, however, did inhibit induction of the G2/M markers *Ccnb2* and *Foxm1* mRNAs, as well as *G6pd*, *Gbe1* and *Me1* mRNAs in these cells (Fig. S4H). AKTi treatment also inhibited induction of *Me1, Gbe1, Taldo1 and Tkt* mRNAs in Adv-p62 infected primary hepatocytes (Fig. S4I). In conclusion, AKT activation accounts for most, if not all, of the metabolic effects of NRF2 activation, and NRF2-induced AKT activation and hepatomegaly can be separated from the NRF2-activated antioxidant response.

NRF2 transcriptionally controls EGFR and PDGFR ligand expression

We used immunohistochemistry to spatially define the effect of p62 overexpression and NRF2 activation on EGFR expression and phosphorylation. Both Adv-p62 transduction and *Nrf2*$^{Act\text{-}hep}$ activation decreased hepatocyte staining with an antibody to non-phosphorylated EGFR but increased staining with a phospho-EGFR antibody (Fig. 5A and Fig. S5A). These results suggested that total EGFR downregulation was probably due to its chronic activation, which triggers receptor endocytosis.[43,44] Consistent with its effects on receptor tyrosine phosphorylation, p62 WT, but not p62KIR^{-}, increased mRNAs for the EGFR ligands *Tgfa* and amphiregulin (*Areg*) and PDGFR ligands *Pdgfa*, *Pdgfb* and *Pdgfc* (Fig. 5B and Fig. S5B). It also increased expression of mRNAs for the ligand processing enzymes *Adam10* and *Adam17* (Fig. 5C). Elevated *Tgfa, Areg* and *Egf* mRNA expression was also seen in *Nrf2*$^{Act\text{-}hep}$ livers, while *Egfr* mRNA amounts were unchanged (Fig. 5D). *Pdgfc* mRNA was also elevated in the *Nrf2*$^{Act\text{-}hep}$ livers (Fig. S5C). Neither p62 overexpression nor NRF2 activation led to induction of *Igf1*, *Igf2*, *Igf1r* or *Igf2r* mRNAs (Fig. S5D).

To validate that these p62-dependent changes in growth factor receptor protein expression are cell autonomous, we overexpressed p62 WT and p62KIR^{-} in primary mouse hepatocytes in the absence of non-parenchymal cells. As in intact liver, Adv-p62 induced downregulation of EGFR and PDGFRα and stimulated AKT T308 phosphorylation (Fig. S5E). Adv-p62 also induced the classic NRF2 target genes *Slc7a11, Nqo1* and *Gclc* as well as the metabolic genes *Gbe1, Me1, Taldo1* and *Tkt* and the growth factors *Areg*, *Tgfa*, and *Pdgfc*, none of which were strongly upregulated in Adv-p62 infected *Nrf2*$^{-/-}$ hepatocytes (Fig. S5F). These results confirm that NRF2 is required for induction of all these genes.

Recent studies had suggested possible transcriptional regulation of *Pdgfa* mRNA by NRF2.[45] To determine whether other *Pdgf* and *Egf* family member genes are directly activated by NRF2, we conducted chromatin immunoprecipitation (ChIP) experiments on primary hepatocytes from *Nrf2*$^{Act\text{-}hep}$ mice, as well as hepatocytes transduced with Adv-GFP or Adv-p62. These experiments confirmed recruitment of NRF2 to the *Tgfa* and *Areg* gene promoters as well as to the *Pdgfc* and *Nqo1* promoters (Fig. 5E,F). Primary hepatocytes isolated from *Nrf2*$^{-/-}$ mice, used as controls, did not show any significant signal above the non-specific antibody control.

To validate NRF2-mediated growth factor induction, we collected conditioned medium (CM) from Adv-GFP and Adv-p62 infected primary hepatocytes and added it to non-infected hepatocytes. As early as 15 min after its addition, CM from Adv-p62-transduced hepatocytes induced phosphorylation of EGFR, PDGFRα and AKT (Fig. 6A). CM from primary *Nrf2*$^{Act\text{-}hep}$ hepatocytes, but not primary WT hepatocyte CM, also induced AKT phosphorylation and EGFR downregulation 24 h after its addition (Fig. S6A). Growth factor-mediated receptor phosphorylation and activation are accompanied by endocytic trafficking. CM from Adv-p62-transduced hepatocytes induced EGFR internalization at 15 min after its addition (Fig. 6B). To confirm that growth factors present in the CM activate EGFR and PDGFR, mouse primary hepatocytes were treated with recombinant EGF and PDGFAA. At 15 min, EGF and PDGFAA induced EGFR and PDGFR phosphorylation as well as AKT phosphorylation and EGFR downregulation (Fig. 6C). These effects were blocked by the specific EGFR inhibitor erlotinib and the PDGFR inhibitor CP-673451.

Erlotinib and CP-673451 also blocked AKT and EGFR phosphorylation in response to Adv-p62 CM (Fig. 6D). Consistent with these *in vitro* findings, treatment of Adv-p62-transduced mice with the broad-spectrum tyrosine kinase inhibitor dasatinib, the EGFR inhibitor erlotinib and the PDGFR inhibitor CP-673451 alone or in combination reduced the extent of hepatomegaly (Fig. S6B,C), and they consistently inhibited phosphorylation of AKT as well as EGFR and PDGFR downregulation (Fig. S6D). Combined treatment with erlotinib and CP-673451 also inhibited Adv-p62-induced upregulation of *Me1*, *Gbe1* and *Taldo1* mRNAs (Fig. 6E) as well as liver glycogen accumulation (Fig. S6E). The residual degree of liver PAS staining in erlotinib + CP-673451 treated mice was similar to that seen after transduction of $Nrf2^{-/-}$ mice with Adv-p62. Erlotinib treatment of primary $Nrf2^{Act\text{-}hep}$ hepatocytes inhibited induction of *Ccnb2* and *Foxm1* as well as *G6pd, Gbe1, Me1* and *Fasn* mRNAs (Fig. S6F).

Autophagy disruption causes NRF2-mediated growth factor expression and AKT activation

Liver-specific ATG7 ablation causes hepatomegaly through p62-mediated NRF2 activation.[12–15] NRF2 activation in autophagy-deficient livers is prevented by p62 deletion.[14] To determine whether NRF2 activation also mediates growth factor expression and AKT activation in the autophagy-deficient liver, we used the Alb-Cre driver to generate $Atg7^{\Delta hep}$ and $Atg7^{\Delta hep}; p62^{\Delta hep}$ mice. Consistent with results obtained from Mx-1-Cre mediated *Atg7* ablation and whole body *p62* knockout ($p62^{-/-}$) mice,[14] parenchymal cell-specific p62 ablation completely prevented hepatomegaly caused by *Atg7* ablation (Fig. 7A). Like other drivers of NRF2 activation, *Atg7* ablation led to AKT T308, PDK1 S241 and FOXO1 S256 phosphorylation, and EGFR downregulation, along with strong p62 accumulation and NQO1 expression (Fig. 7B). All of these effects were reversed upon p62 ablation. Like p62 overexpressing mice, $Atg7^{\Delta hep}$ mice displayed elevated liver expression of *Tgfa, Areg, Pdgfb* and *Pdgfc* mRNAs, all of which were reduced upon parenchymal cell-specific p62 ablation (Fig. 7C). Liver-specific *Atg7* ablation also resulted in upregulation of *Slc7a11, Cd36*, *Ccnb2, G6pd*, *Aurka*, *Mthfd2, Nqo1, Tkt, Taldo1,* and *HK2* transcripts, all of which were diminished after p62 ablation (Fig. S7A). As seen in Adv-p62-transduced livers, *Atg7* ablation increased liver *Adam10* and *Adam17* mRNAs and this was also reduced by p62 ablation (Fig. S7B). *Atg7* deletion increased liver TG accumulation and this was reversed by p62 ablation (Fig. S7C).

Human hepatomegaly specimens show NRF2 and EGFR activation

To determine whether some types of human hepatomegaly are associated with NRF2 activation and EGFR engagement, we analyzed liver specimens collected from patients with pyrrolizidine alkaloid-induced HSOS. HSOS is a potentially life-threatening hepatic vascular disease associated with abdominal distension, pain in the hepatic region, ascites, jaundice and hepatomegaly. Compared with normal livers, livers from patients with HSOS and AIH showed increased expression of p62 and the NRF2 target NQO1 (Fig. 8 and Fig. S8). In addition, livers from patients with HSOS and AIH showed downregulation of non-phosphorylated EGFR and upregulation of Tyr1068 phosphorylated EGFR and Ser473 phosphorylated AKT, as well as glycogen accumulation.

Discussion

Hepatomegaly can be triggered by insulin overdosing and many other etiologies that cause dysregulated glycogen or lipid accumulation in hepatocytes.[8] Other than insulin overdosing, the mechanisms underlying hepatomegaly were unknown, although hepatomegaly in the autophagy-deficient liver was attributed to NRF2 activation.[12,13] Originally recognized as the master activator of the anti-oxidant response,[19] NRF2 is also an important metabolic regulator that redirects glucose and glutamine into the anabolic pentose phosphate pathway in cancer cells with constitutive PI3K-AKT signaling.[28] This function of NRF2, however, was suggested to take place only in proliferative cell types, and not in adult liver, whose cells do not proliferate. Here we show that in addition to being responsive to AKT signaling,[28] NRF2 itself is a potent, but indirect, activator of AKT in non-transformed and non-proliferative hepatocytes that are subjected to either mutational NRF2 activation, p62 overexpression, or disruption of autophagy. Importantly, our results reveal a missing link between NRF2 activation and hepatomegaly, that depends on AKTactivation. AKT activation may also underlie other forms of hepatomegaly. Quite surprisingly, we found that the effects of NRF2 on hepatic glucose and lipid metabolism are all depedent on AKT activation. Mechanistically, we show that in non-transformed and non-proliferative hepatocytes, NRF2 directly activates transcription of growth factor genes encoding the PDGFR ligand *Pdgfc* and the EGFR ligands *Tgfa* and *Areg*, which contribute to AKT activation via autocrine signaling. These findings suggest that any condition that leads to hepatic NRF2 activation, including autophagy disruption and oxidative stress, as well as chronic insulin or growth factor receptor activation, can culminate in hepatomegaly, a common pathology that, at least in mice, is fully reversed on AKT or EGFR + PDGFR inhibition. Through the use of such inhibitors, we separated the metabolic effects of NRF2 from its cytoprotective anti-oxidant response, which is growth factor and AKT independent.

Although a recent study carried out in oxidatively stressed *Kras*-transformed pancreatic cancer cells that were completely depleted of NRF2, suggested a link between NRF2 and EGFR signaling, this link was attributed to indirect redox regulation of ADAM10, as well as redox control of cap-dependent translation.[31] However, it should be emphasized that other than deliberate *Nfe2l2* gene disruption, a complete NRF2 deficiency does not occur in cancer cells, many of which show elevated NRF2 expression and activity.[23] Additionally, a proteomic screen for NRF2-dependent drug targets in lung cancer cells did not substantiate a link between NRF2 and the translational machinery.[46] Of further note, in whole body $Nfe2l2^{-/-}$ mice, liver and pancreatic development are totally normal as long as the mice are not subjected to extreme oxidative stress.[26] Thus, a complete NRF2 deficiency does not impact EGFR signaling, which is needed for proper liver development.[47] Although we investigated the function of NRF2 in non-transformed liver parenchymal cells, the results thus obtained may also be relevant to cancers in which NRF2 is mutationally activated.

Genetic studies of autophagy-deficient mice suggested a key role for the p62-KEAP1-NRF2 axis in driving both hepatomegaly and liver injury.[13–15] Interestingly, NRF2 activation rather than p62 accumulation seems to be the dominant cause of hepatomegaly and tissue injury in the autophagy impaired liver, as NRF2 deletion completely rescued liver damage and hepatomegaly in $Atg7^{\Delta hep}; Keap1^{\Delta hep}$ mice.[12] Consistent with previous results obtained in

Atg7$^{F/F}$*; Mx-1-Cre and Atg7*$^{F/F}$*; Mx-1-Cre; p62*$^{-/-}$ mice,[13] our liver-specific p62 deletion also rescued hepatomegaly in *Atg7*$^{\Delta hep}$ mice. However, in contrast to previous reports,[12,13,25,33] we found that persistent NRF2 activation in *Nrf2*$^{Act\text{-}hep}$ mice caused hepatomegaly without any sign of liver injury. Likewise, adenovirus-mediated p62 overexpression led to hepatomegaly, but did not induce liver damage, although ectopic expression of p62 in growing cell lines was reported to cause cytotoxicity due to inhibition of ubiquitin-dependent proteolysis.[48] In respect to hepatomegaly and liver injury, the liver phenotype of the *Nrf2*$^{Act\text{-}hep}$ mouse is very similar to that of the *Keap1*$^{\Delta hep}$ mouse.[13,49] Curiously, a different mouse line with liver constitutively active NRF2, in which the 88 N-terminal residues including the KEAP1 binding site were deleted, did not show any hepatomegaly.[50] The discrepancy between the 2 mouse strains is likely due to differences in NRF2 expression levels, as the previous study used NRF2 heterozygotes, whereas our study was conducted in homozygote mutant mice. In addition, hepatomegaly in *Nrf2*$^{Act\text{-}hep}$ mice is not as severe as in *Atg7*$^{\Delta hep}$ mice. Unlike the *Atg7*$^{\Delta hep}$ mice, in which the liver continued to grow for many months, hepatomegaly in *Nrf2*$^{Act\text{-}hep}$ mice peaked at 8 weeks of age. Furthermore, the milder hepatomegaly in *Nrf2*$^{Act\text{-}hep}$ mice did not disrupt the normal liver structure and did not cause narrowing of sinusoidal capillaries or abnormal morphology of bile canaliculi, all of which can cause liver injury.

Through its ability to activate AKT downstream of EGFR and PDGFR, NRF2 leads to induction of a large battery of anabolic genes that convert glucose to glycogen and glucose-derived acetyl-CoA to fatty acids, resulting in decreased blood glucose and increased liver glycogen and triglycerides. Our findings of increased triglyceride accumulation in 8 month-old *Atg7*$^{\Delta hep}$ mice differ from a recent report that 5 week-old mouse hepatocytes lacking ATG7 show decreased lipid droplet biosynthesis.[51] The main difference between this prior study and ours is the age of the mice. In addition, our results are consistent with a previous report based on analysis of 4 month-old *Atg7*$^{\Delta hep}$ mice.[52] Consistent with its ability to prevent NRF2 activation, p62 deletion abolished triglyceride accumulation in *Atg7*$^{\Delta hep}$ mice. Although the liver hardly contains any proliferative cells, AKT activation also causes upregulation of p21 and G2/M markers, suggesting that it favors an increase in cell size (hypertrophy) over cell number (hyperplasia). Examination of human clinical specimens confirms p62 accumulation, NRF2 activation and elevated EGFR and AKT phosphorylation in at least one form of human hepatomegaly caused by toxicant exposure – pyrrolizidine alkaloid-induced HSOS.[53] Pyrrolizidine alkaloids undergo metabolic activation in hepatocytes to form highly reactive metabolites that generate pyrrole-protein adducts.[54] This reaction may interfere with the autophagic degradation of p62 and lead to KEAP1 inactivation, thereby causing strong NRF2 activation, which eventually culminates in AKT-driven hepatomegaly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

We thank eBioscience, Cell Signaling Technologies, Santa Cruz Technologies and Promega for gifts of reagents. We thank Randal Kaufman, Su Hua and Elsa Sanchez-Lopez for advice and helpful suggestions. We also thank Elijah Hatfield for editorial assistance.

Financial support

This research was supported by the Superfund Basic Research Program (P42-ES010337), NIH (P01-DK098108, 5R01DK120714–02, R01-CA118165, R01CA198103, R37-AI043477, P01-CA128814, R01-CA211794) and a C3 Pedal the Cause grant to M.K., who holds the Ben and Wanda Hildyard Chair for Mitochondrial and Metabolic Diseases; The NIH also supported M.T.D.-M. (R01-CA192642) and J.M. (R01-DK108743 and R01-CA211794). Research at Nanjing University was supported by the National Key Research and Development Program of China Grant (2016YFC0905900 to B.S.), the State Key Program of National Natural Science Foundation Grant (81430062 to B.S.) and the Innovative Research Team Grant of Jiangsu Province (303073227 to B.S.). F.H. was supported by the Eli Lilly LIFA program; K.T. was supported by JSPS KAKENHI (JP15K21775) and the “Kibou Projects” Startup Support for Young Researchers in Immunology; A.U. was supported by Bristol-Myers Squibb and was supported by JSPS KAKENHI Grant #19K08377, AMED Grant #JP19fk0210059.

Abbreviations

ACC1	acetyl-CoA carboxylase 1
Adv	adenovirus
AIH	autoimmune hepatitis
BW	body weight
ChIP	chromatin immunoprecipitation
CM	conditioned medium
DNL	*de novo* lipogenesis
EGF	epidermal growth factor
EGFR	EGF receptor
FASN	fatty acid synthase
FOXO	forkhead box O1
G6P	glucose 6 phosphate dehydrogenase
GBE1	glycogen branching enzyme 1
GCK	glucokinase
GCLC	glutamate-cysteine ligase catalytic
GSEA	gene set enrichment analysis
GSK	glycogen synthase kinase
HK2	hexokinase 2
HMF	high magnification field

HSOS	hepatic sinusoidal obstruction syndrome
IGF	insulin-like growth factor
KEAP1	Kelch-like ECH-associated protein 1
NRF2	nuclear factor erythroid 2-related factor 2
ME1	malic enzyme
MTHFD2	methylenetetrahydrofolate dehydrogenase 2
PDGF	platelet-derived growth factor
PDGFR	PDGF receptor
PPAT	phosphoribosyl pyrophosphate amidotransferase
RLU	relative light units
ROS	reactive oxygen species
SCD1	stearoyl-CoA desaturase 1
SLC7A11	solute carrier family 7 member 11
SREBP1c	sterol regulatory element-binding protein 1c
TALDO	transaldolase 1
TKT	transketolase
WT	wild-type

References

[1]. Metze D, Cury VF, Gomez RS, Marco L, Robinson D, Melamed E, et al. Hepatomegaly. Encyclopedia of Molecular Mechanisms of Disease. Berlin, Heidelberg: Springer Berlin Heidelberg; 2009. p. 824–825.

[2]. Ebert EC, Nagar M, Hagspiel KD. Gastrointestinal and hepatic complications of sickle cell disease. Clin Gastroenterol Hepatol 2010;8:483–489. [PubMed: 20215064]

[3]. Kwok MK, Lewin KJ. Massive hepatomegaly in adult polycystic liver disease. Am J Surg Pathol 1988;12:321–324. [PubMed: 3281482]

[4]. Murakami J, Shimizu Y. Hepatic manifestations in hematological disorders. Int J Hepatol 2013;2013:484903. [PubMed: 23606974]

[5]. Froesch ER, Wolf HP, Baitsch H, Prader A, Labhart A. Hereditary fructose intolerance: an inborn defect of hepatic fructose-1-phosphate splitting aldolase. Am J Med 1963;34:151–167. [PubMed: 13959929]

[6]. Bonnefont JP, Demaugre F, Prip-Buus C, Saudubray JM, Brivet M, Abadi N, et al. Carnitine palmitoyltransferase deficiencies. Mol Genet Metab 1999;68:424–440. [PubMed: 10607472]

[7]. Asherov J, Mimouni M, Varsano I, Lubin E, Laron Z. Hepatomegaly due to self-induced hyperinsulinism. Arch Dis Child 1979;54:148–149. [PubMed: 444328]

[8]. Chatila R, West AB. Hepatomegaly and abnormal liver tests due to glycogenosis in adults with diabetes. Medicine 1996;75:327–333. [PubMed: 8982149]

[9]. Hay N Interplay between FOXO, TOR, and Akt. Biochim Biophys Acta 2011;1813:1965–1970. [PubMed: 21440577]

[10]. Ono H, Shimano H, Katagiri H, Yahagi N, Sakoda H, Onishi Y, et al. Hepatic Akt activation induces marked hypoglycemia, hepatomegaly, and hypertriglyceridemia with sterol regulatory element binding protein involvement. Diabetes 2003;52:2905–2913. [PubMed: 14633850]

[11]. Ni HM, Chao X, Yang H, Deng F, Wang S, Bai Q, et al. Dual roles of mammalian target of rapamycin in regulating liver injury and tumorigenesis in autophagy-defective mouse liver. Hepatology 2019;70(6): 2142–2155. [PubMed: 31095752]

[12]. Taguchi K, Fujikawa N, Komatsu M, Ishii T, Unno M, Akaike T, et al. Keap1 degradation by autophagy for the maintenance of redox homeostasis. Proc Natl Acad Sci U S A 2012;109:13561–13566. [PubMed: 22872865]

[13]. Komatsu M, Kurokawa H, Waguri S, Taguchi K, Kobayashi A, Ichimura Y, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat Cell Biol 2010;12:213–223. [PubMed: 20173742]

[14]. Komatsu M, Waguri S, Koike M, Sou YS, Ueno T, Hara T, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. Cell 2007;131:1149–1163. [PubMed: 18083104]

[15]. Komatsu M, Waguri S, Ueno T, Iwata J, Murata S, Tanida I, et al. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol 2005;169:425–434. [PubMed: 15866887]

[16]. Ni HM, Woolbright BL, Williams J, Copple B, Cui W, Luyendyk JP, et al. Nrf2 promotes the development of fibrosis and tumorigenesis in mice with defective hepatic autophagy. J Hepatol 2014;61:617–625. [PubMed: 24815875]

[17]. Moscat J, Karin M, Diaz-Meco MT. p62 in cancer: signaling adaptor beyond autophagy. Cell 2016;167:606–609. [PubMed: 27768885]

[18]. Yamamoto M, Kensler TW, Motohashi H. The KEAP1-NRF2 system: a thiol-based sensor-effector apparatus for maintaining redox homeostasis. Physiol Rev 2018;98:1169–1203. [PubMed: 29717933]

[19]. Nguyen T, Nioi P, Pickett CB. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 2009;284:13291–13295. [PubMed: 19182219]

[20]. Shibata T, Ohta T, Tong KI, Kokubu A, Odogawa R, Tsuta K, et al. Cancer related mutations in NRF2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy. Proc Natl Acad Sci 2008;105:13568–13573. [PubMed: 18757741]

[21]. Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad I Ben, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012;44:694–698. [PubMed: 22561517]

[22]. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev 2013;27:2179–2191. [PubMed: 24142871]

[23]. Taguchi K, Yamamoto M. The KEAP1–NRF2 system in cancer. Front Oncol 2017;7:85. [PubMed: 28523248]

[24]. Lee YA, Noon LA, Akat KM, Ybanez MD, Lee TF, Berres ML, et al. Autophagy is a gatekeeper of hepatic differentiation and carcinogenesis by controlling the degradation of Yap. Nat Commun 2018;9:49642.

[25]. Khambu B, Li T, Yan S, Yu C, Chen X, Goheen M, et al. Hepatic autophagy deficiency compromises farnesoid X receptor functionality and causes cholestatic injury. Hepatology 2019;69:2196–2213. [PubMed: 30520052]

[26]. Enomoto A, Itoh K, Nagayoshi E, Haruta J, Kimura T, O'Connor T, et al. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 2001;59:169–177. [PubMed: 11134556]

[27]. Beyer TA, Xu W, Teupser D, auf dem Keller U, Bugnon P, Hildt E, et al. Impaired liver regeneration in Nrf2 knockout mice: role of ROS-mediated insulin/IGF-1 resistance. EMBO J 2008;27:212–223. [PubMed: 18059474]

[28]. Mitsuishi Y, Taguchi K, Kawatani Y, Shibata T, Nukiwa T, Aburatani H, et al. Nrf2 redirects glucose and glutamine into anabolic pathways in metabolic reprogramming. Cancer Cell 2012;22:66–79. [PubMed: 22789539]

[29]. Rojo de la Vega M, Chapman E, Zhang DD. NRF2 and the hallmarks of cancer. Cancer Cell 2018;34:21–43. [PubMed: 29731393]

[30]. Todoric J, Antonucci L, Di Caro G, Li N, Wu X, Lytle NKK, et al. Stress-activated NRF2-MDM2 cascade controls neoplastic progression in pancreas. Cancer Cell 2017;32:824–839.e8. [PubMed: 29153842]

[31]. Chio IIC, Jafarnejad SM, Ponz-Sarvise M, Park Y, Rivera K, Palm W, et al. NRF2 promotes tumor maintenance by modulating mRNA translation in pancreatic cancer. Cell 2016;166:963–976. [PubMed: 27477511]

[32]. Umemura A, He F, Taniguchi K, Nakagawa H, Yamachika S, Font-Burgada J, et al. p62, upregulated during preneoplasia, induces hepatocellular carcinogenesis by maintaining survival of stressed HCC-initiating cells. Cancer Cell 2016;29:935–948. [PubMed: 27211490]

[33]. Khambu B, Huda N, Chen X, Antoine DJ, Li Y, Dai G, et al. HMGB1 promotes ductular reaction and tumorigenesis in autophagy-deficient livers. J Clin Invest 2018;128:2419–2435. [PubMed: 29558368]

[34]. Taniguchi K, Yamachika S, He F, Karin M. p62/SQSTM1-Dr. Jekyll and Mr. Hyde that prevents oxidative stress but promotes liver cancer. FEBS Lett 2016;590:2375–2397. [PubMed: 27404485]

[35]. Park JM, Greten FR, Li ZW, Karin M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science 2002;297:2048–2051. [PubMed: 12202685]

[36]. Bentzinger CF, Romanino K, Cloëtta D, Lin S, Mascarenhas JB, Oliveri F, et al. Skeletal muscle-specific ablation of raptor, but not of rictor, causes metabolic changes and results in muscle dystrophy. Cell Metab 2008;8:411–424. [PubMed: 19046572]

[37]. Müller TD, Lee SJ, Jastroch M, Kabra D, Stemmer K, Aichler M, et al. p62 links β-adrenergic input to mitochondrial function and thermogenesis. J Clin Invest 2013;123:469–478. [PubMed: 23257354]

[38]. Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci 1996;93:13943–13948. [PubMed: 8943040]

[39]. Zhong Z, Umemura A, Sanchez-Lopez E, Liang S, Shalapour S, Wong J, et al. NF-κB restricts inflammasome activation via elimination of damaged mitochondria. Cell 2016;164:896–910. [PubMed: 26919428]

[40]. Mullany LK, Nelsen CJ, Hanse EA, Goggin MM, Anttila CK, Peterson M, et al. Akt-mediated liver growth promotes induction of cyclin E through a novel translational mechanism and a p21-mediated cell cycle arrest. J Biol Chem 2007;282:21244–21252. [PubMed: 17517888]

[41]. Pajares M, Cuadrado A, Rojo AI. Modulation of proteostasis by transcription factor NRF2 and impact in neurodegenerative diseases. Redox Biol 2017;11:543–553. [PubMed: 28104575]

[42]. Manning BD, Toker A. AKT/PKB signaling: navigating the network. Cell 2017;169:381–405. [PubMed: 28431241]

[43]. Dikic I Mechanisms controlling EGF receptor endocytosis and degradation. Biochem Soc Trans 2003;31:1178–1181. [PubMed: 14641021]

[44]. Er EE, Mendoza MC, Mackey AM, Rameh LE, Blenis J. AKT facilitates EGFR trafficking and degradation by phosphorylating and activating PIKfyve. Sci Signaling 2013;6:ra45.

[45]. Liu Y, Liu G, Zhang Y, Zou L, Gao Q, Liu D, et al. Activation of AKT pathway by Nrf2/PDGFA feedback loop contributes to HCC progression. Oncotarget 2016;7:65389–65402. [PubMed: 27588483]

[46]. Bar-Peled L, Kemper EK, Suciu RM, Vinogradova EV, Backus KM, Horning BD, et al. Chemical proteomics identifies druggable vulnerabilities in a genetically defined cancer. Cell 2017;171:696–709.e23. [PubMed: 28965760]

[47]. Natarajan A, Wagner B, Sibilia M. The EGF receptor is required for efficient liver regeneration. Proc Natl Acad Sci 2007;104:17081–17086. [PubMed: 17940036]

[48]. Korolchuk VI, Mansilla A, Menzies FM, Rubinsztein DC. Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Mol Cell 2009;33:517–527. [PubMed: 19250912]

[49]. Okawa H, Motohashi H, Kobayashi A, Aburatani H, Kensler TW, Yamamoto M. Hepatocyte-specific deletion of the keap1 gene activates Nrf2 and confers potent resistance against acute drug toxicity. Biochem Biophysical Res Commun 2006;339:79–88.

[50]. Köhler UA, Kurinna S, Schwitter D, Marti A, Schäfer M, Hellerbrand C, et al. Activated Nrf2 impairs liver regeneration in mice by activation of genes involved in cell-cycle control and apoptosis. Hepatology 2014;60:670–678. [PubMed: 24310875]

[51]. Takahashi S, Sou Y-S, Saito T, Kuma A, Yabe T, Koike M, et al. Autophagy controls lipid droplet formation by fine-tuning NCoR1 levels. BioRxiv 2019:722686.

[52]. Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, et al. Autophagy regulates lipid metabolism. Nature 2009;458:1131–1135. [PubMed: 19339967]

[53]. Chojkier M Hepatic sinusoidal-obstruction syndrome: toxicity of pyrrolizidine alkaloids. J Hepatol 2003;39:437–446. [PubMed: 12927933]

[54]. Yang M, Ruan J, Gao H, Li N, Ma J, Xue J, et al. First evidence of pyrrolizidine alkaloid N-oxide-induced hepatic sinusoidal obstruction syndrome in humans. Arch Toxicol 2017;91:3913–3925. [PubMed: 28620673]

Highlights

- Liver p62 accumulation and constitutive NRF2 activation lead to liver lipid buildup, glycogen synthesis and hepatomegaly.
- NRF2 activation mediates transcriptional induction of PDGF and EGF receptor ligands that activate AKT.
- AKT and tyrosine kinase inhibitors block NRF2-mediated AKT activation and hepatomegaly.
- NRF2-AKT signaling is elevated in HSOS- and AIH-related human hepatomegaly.

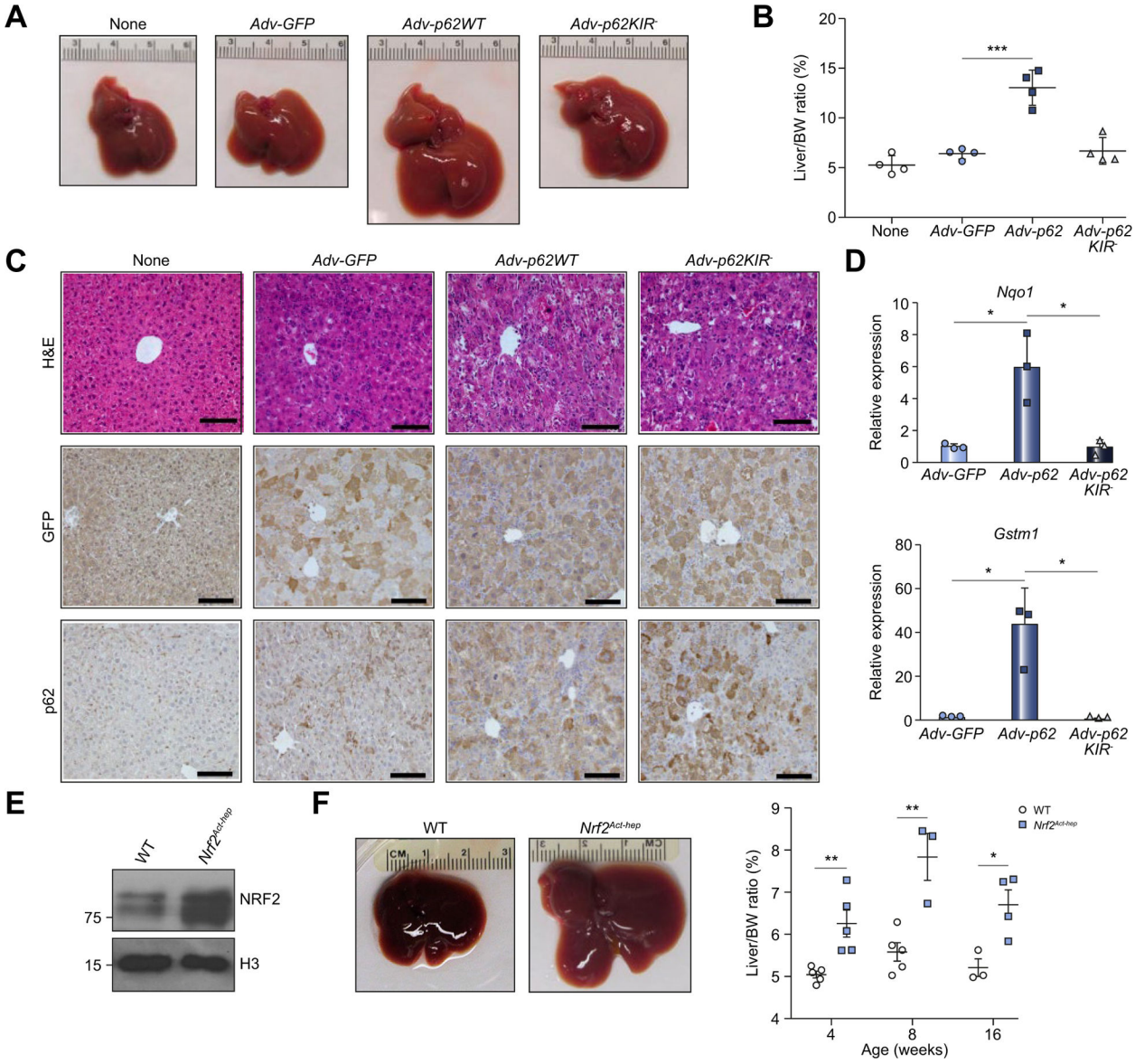


Fig. 1. Liver-specific p62-mediated NRF2 activation leads to hepatomegaly.

(A and B) Macroscopic appearance of livers (A) and liver/BW ratio (in %) (B) before (None) and 7 days after intravenous (i.v.) injection of adenoviruses expressing GFP, p62 WT, or p62KIR^{-} (n = 4/group) into $p62^{\Delta hep}$ mice. (*t* test) (C) H&E, GFP and p62 staining of livers from $p62^{\Delta hep}$ mice treated as above. Scale bars, 100 μm. (D) qPCR analysis of the NRF2 targets *Nqo1* and *Gstm1* (n = 3/group) in above mice. (*t* test) (E) Nuclear NRF2 expression in 8-week-old mice with indicated *Nrf2* genotypes; Histone H3 was used as loading and purity control. (F) Gross morphology of 8-week-old WT and $Nrf2^{Act\text{-}hep}$ livers and liver/BW ratio (in %) of above mice at the indicated time points. (*t* test) Mean ± SD (n = 3–5/group); **p* <0.05, ***p* <0.01, ****p* <0.001. Adv, adenovirus; BW, body weight; WT, wild-type.

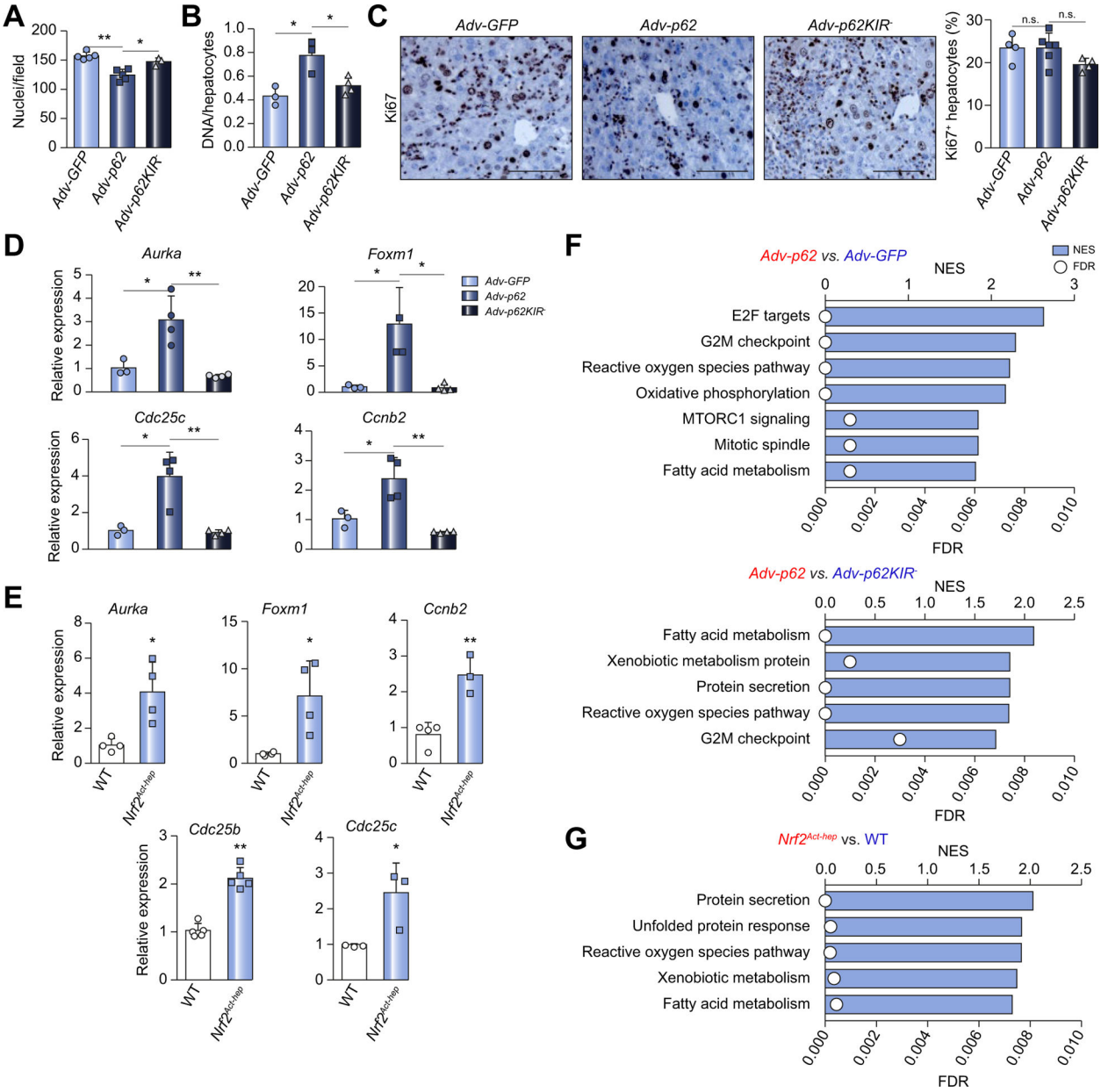


Fig. 2. p62-mediated NRF2 activation causes G2/M arrest and hypertrophy.

(A-C) Number of nuclei per field (A), hepatocyte DNA content (B), Ki67 staining (C left) and quantification of Ki67$^+$ hepatocytes (C right) in $p62^{\Delta hep}$ livers 7 days after i.v. injection of adenoviruses expressing GFP, p62 WT and p62KIR$^-$ (n = 3–6/group). (*t* test) (D) qPCR analysis of G2/M checkpoint genes in livers of $p62^{\Delta hep}$ mice subjected to above treatments (n = 3–4/group). (*t* test) (E) qPCR analysis of G2/M checkpoint markers in WT and $Nrf2^{Act\text{-}hep}$ livers. (*t* test) Mean ± SD (n = 3–5/group). *p <0.05, **p <0.01. (F) GSEA analysis showing that the p62-NRF2 axis activates pathways related to ROS accumulation, G2/M checkpoint, fatty acid metabolism, xenobiotic metabolism and protein secretion. (G) GSEA analysis showing that NRF2 activates pathways related to protein secretion, unfolded protein response (UPR), ROS accumulation, fatty acid metabolism, and xenobiotic

metabolism in livers of *Nrf2*$^{Act\text{-}hep}$ mice relative to WT livers. GSEA, gene set enrichment analysis; ROS, reactive oxygen species; WT, wild-type.

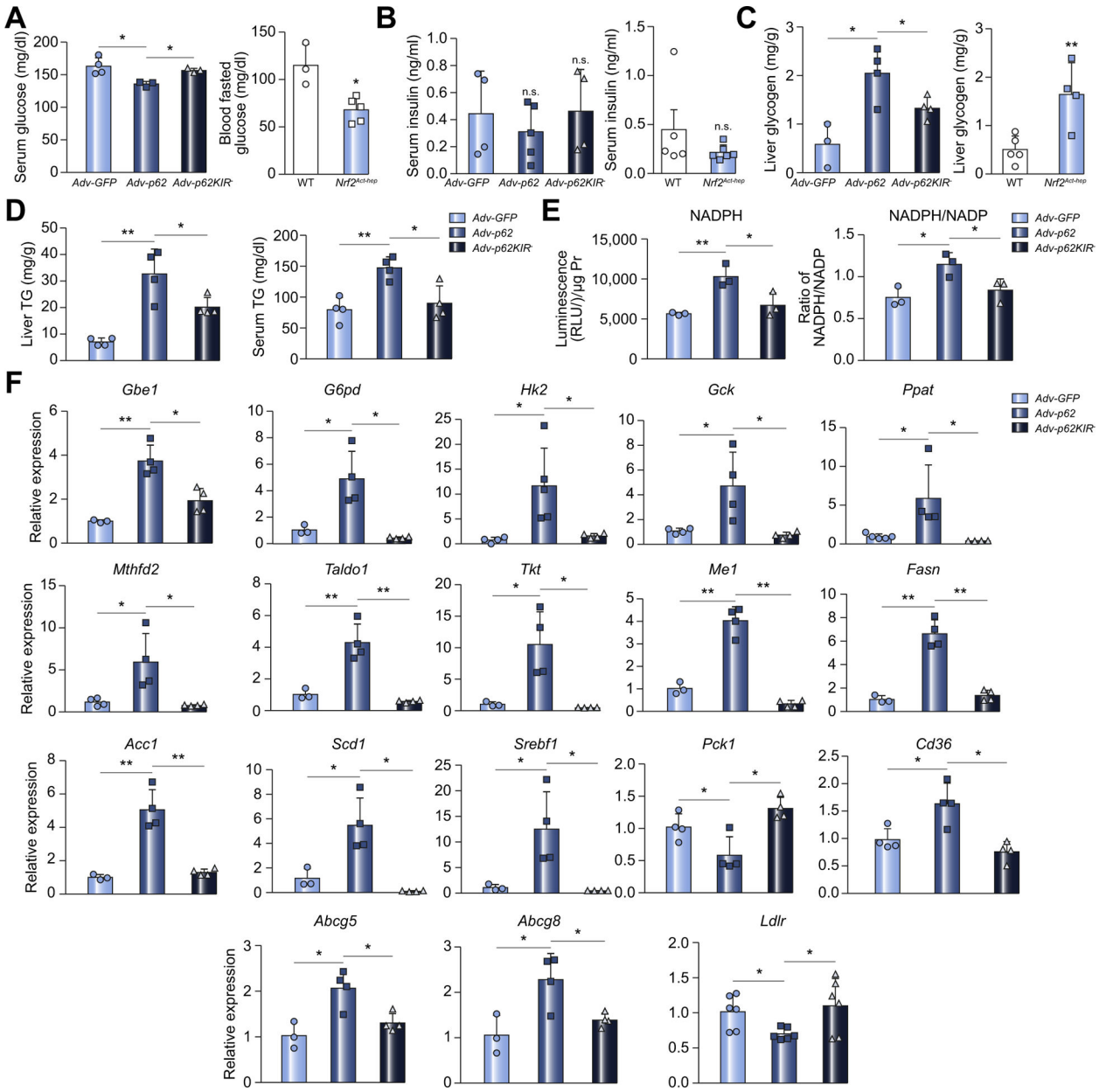


Fig. 3. p62-mediated NRF2 activation causes hypoglycemia, liver glycogen and triglyceride accumulation and upregulation of numerous metabolic enzymes.

Serum or fasting blood glucose (A), serum insulin (B), liver glycogen (C), liver triglycerides and serum triglycerides (D), liver NADPH and NADPH to NADP ratio (E) in $p62^{\Delta hep}$ mice 7 days after i.v. injection of adenoviruses expressing GFP, p62WT or p62KIR^{-} (n = 3–6/group) or WT and $Nrf2^{Act\text{-}hep}$ mice (n = 3–8/group) as indicated. (*t* test) Mean ± SD. (F) Expression of genes involved in glycogen and NADPH production, gluconeogenesis, glycolysis, nucleotide synthesis, *de novo* lipogenesis and lipid uptake was measured by qPCR. (*t* test) Mean ± SD (n = 3–6/group). n.s., not significant, *p <0.05, **p <0.01. RLU, relative light units; WT, wild-type.

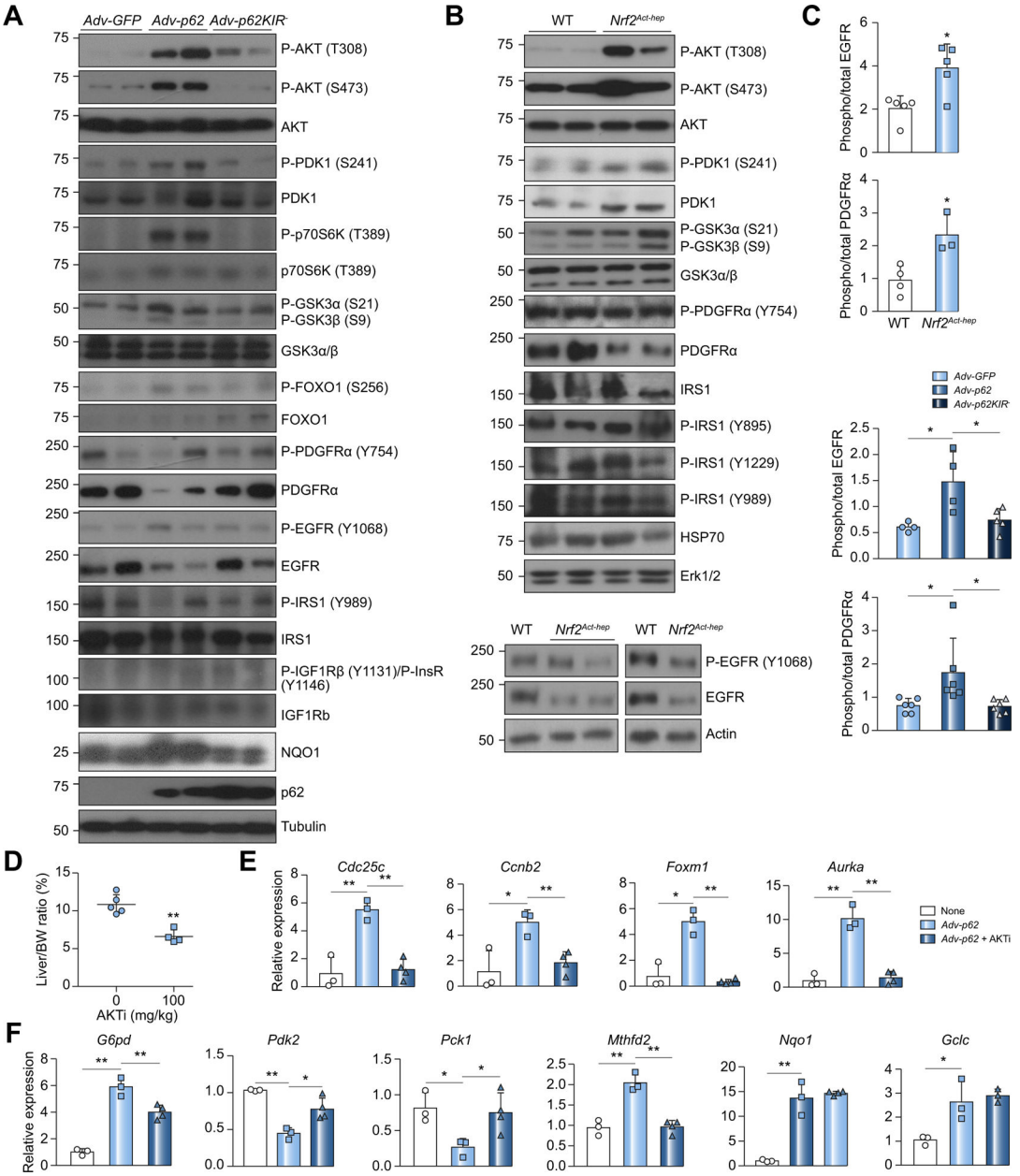


Fig. 4. AKT activation mediates hepatomegaly and induces markers of G2/M arrest.

(A) Immunoblot analysis of liver lysates from GFP-, p62 WT- or p62KIR⁻-transduced *p62*$^{\Delta hep}$ mice. P – phospho. (B) Immunoblot analysis of liver lysates from 8-week-old mice with indicated *Nrf2* genotypes. (C) Densitometry of total and phosphorylated EGFR and PDGFRα from panels A and B. (*t* test) (D) Liver/BW ratio (in %) of WT mice injected with Adv-p62 with or without AKTi treatment. Mean ± SD (n = 4–5/group). (*t* test) (E) qPCR analysis of G2/M checkpoint markers in livers of WT mice subjected to the same treatments as in D. Mean ± SD (n=3–4/group). (*t* test) (F) qPCR analysis of genes involved in metabolism and redox homeostasis in livers of WT mice transduced with Adv-p62 with or without AKTi treatment. (n = 3–4/group). (*t* test) **p* <0.05, ***p* <0.01. Adv, adenovirus; BW, body weight; WT, wild-type.

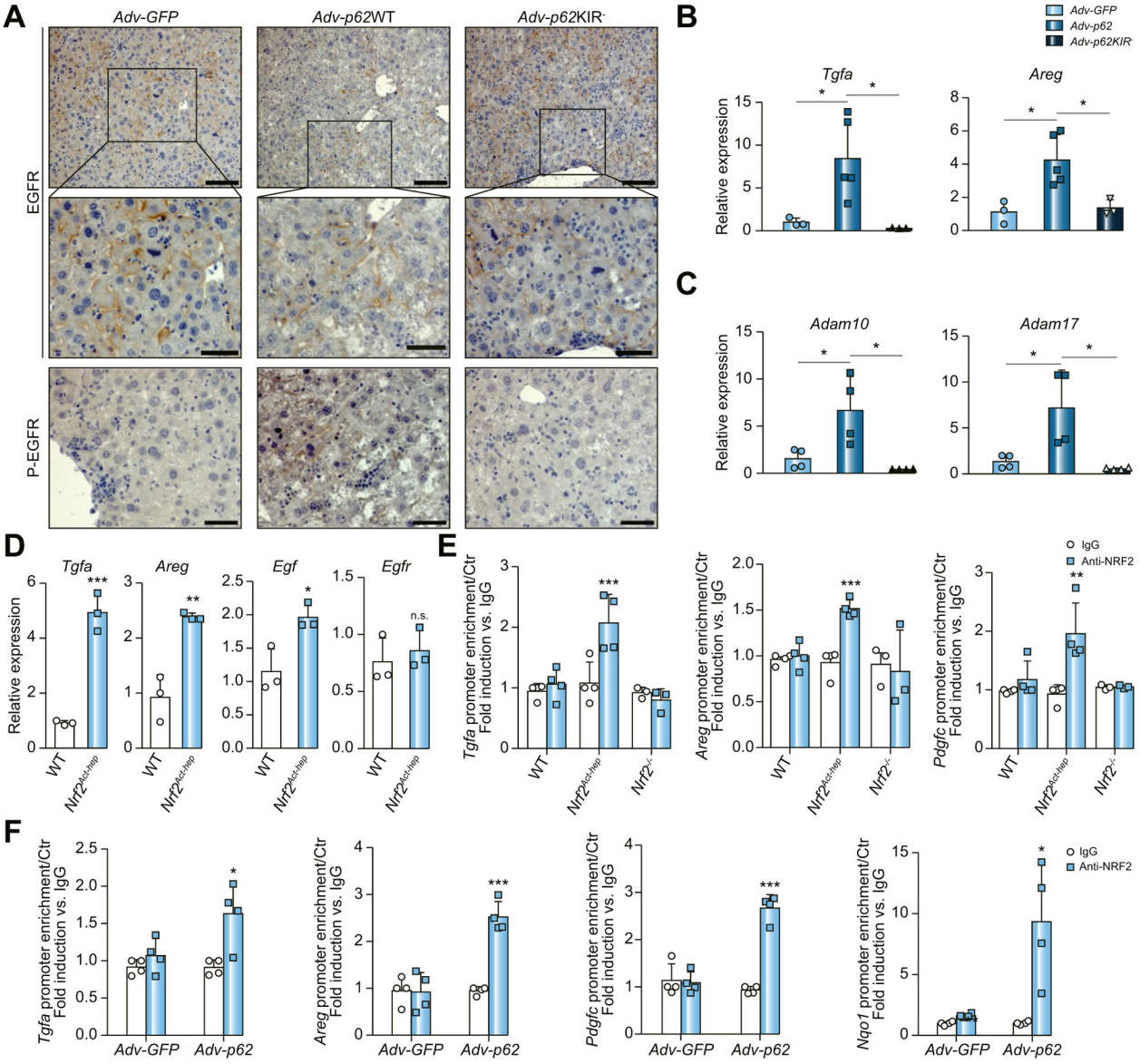


Fig. 5. NRF2 activates EGFR and induces growth factor gene transcription.

(A) Total and Tyr1068 phosphorylated EGFR staining in livers of $p62^{\Delta hep}$ mice transduced with GFP, p62 WT or p62KIR^{-} adenoviruses. Scale bars: 100 μm (top), 50 μm (middle and bottom). (B-D) Expression of genes involved in growth factor signaling was measured by qPCR in livers of above mice and in WT and $Nrf2^{Act\text{-}hep}$ mice. Mean ± SD (n = 3–5/group). (*t* test) (E-F) ChIP assays probing NRF2 recruitment to the *Tgfa*, *Areg*, *Pdgfc* and *Nqo1* promoters in primary hepatocytes isolated from WT, $Nrf2^{Act\text{-}hep}$ and $Nrf2^{-/-}$ mice (E) or primary hepatocytes transduced with Adv-GFP or Adv-p62 for 30 hrs (F). Mean ± SD (n = 3–4). (*t* test) n.s., not significant, **p* <0.05, ***p* <0.01, ****p* <0.001. Adv, adenovirus; ChIP, chromatin immunoprecipitation; WT, wild-type.

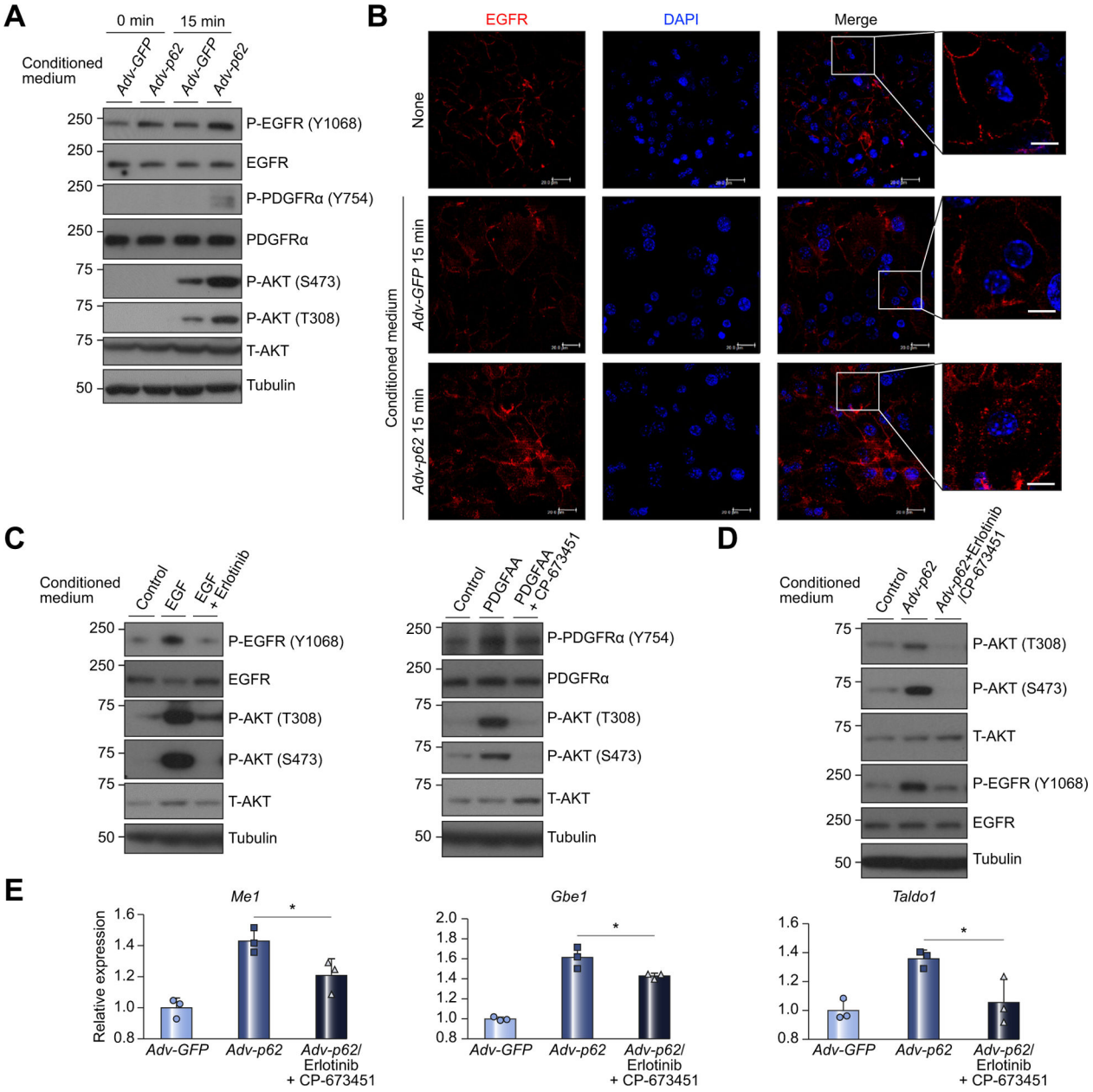


Fig. 6. The p62-NRF2 module triggers growth factor signaling in primary hepatocytes.

(A) Immunblot analysis of primary hepatocytes incubated for 0 or 15 min with conditioned medium from Adv-GFP- or Adv-p62-infected hepatocytes. (B) EGFR immunofluorescence in primary hepatocytes incubated for 15 min with conditioned medium prepared as above. Scale bars: 20 μm (left), 10 μm (right). (C) IB analysis of primary hepatocytes treated with EGF (left) and PDGFAA (right) with or without EGFR and PDGFR inhibitors. (D) Immunoblot analysis of primary hepatocytes treated with Adv-p62 conditioned medium with or without EGFR and PDGFR inhibitors. (E) Expression of metabolic genes in Adv-p62 infected primary hepatocytes treated with or without EGFRi and PDGFRi. Mean ± SD (n = 3). (*t* test) **p* <0.05. Adv, adenovirus; WT, wild-type.

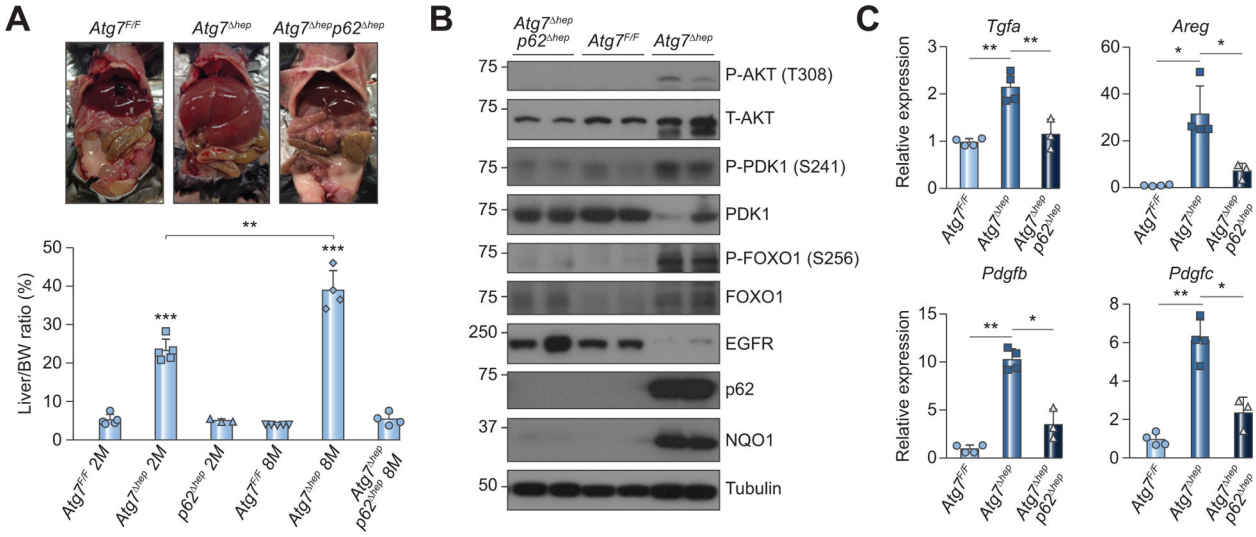


Fig. 7. The p62-NRF2-EGFR module is activated in the ATG7-deficient liver.

(A) Macroscopic appearance of livers of 2-month-old *Atg7*$^{F/F}$ and *Atg7*$^{\Delta hep}$ mice and liver/BW ratio (in %) of 2- and 8-month-old *Atg7*$^{F/F}$, *Atg7*$^{\Delta hep}$ and *Atg7*$^{\Delta hep}$;*p62*$^{\Delta hep}$ mice (n = 3–5/group), $^{**}p < 0.01$, $^{***}p < 0.001$ (*vs.* age-matched *Atg7*$^{F/F}$ mice). (*t* test) (B) Immunoblot analysis of growth factor and AKT signaling in 8-month-old *Atg7*$^{F/F}$, *Atg7*$^{\Delta hep}$, and *Atg7*$^{\Delta hep}$;*p62*$^{\Delta hep}$ mice. (C) qPCR analysis of mRNAs related to growth factor signaling in livers of *Atg7*$^{F/F}$, *Atg7*$^{\Delta hep}$, *Atg7*$^{\Delta hep}$;*p62*$^{\Delta hep}$ mice. Mean ± SD (n = 3–4/group). (*t* test) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. BW, body weight.

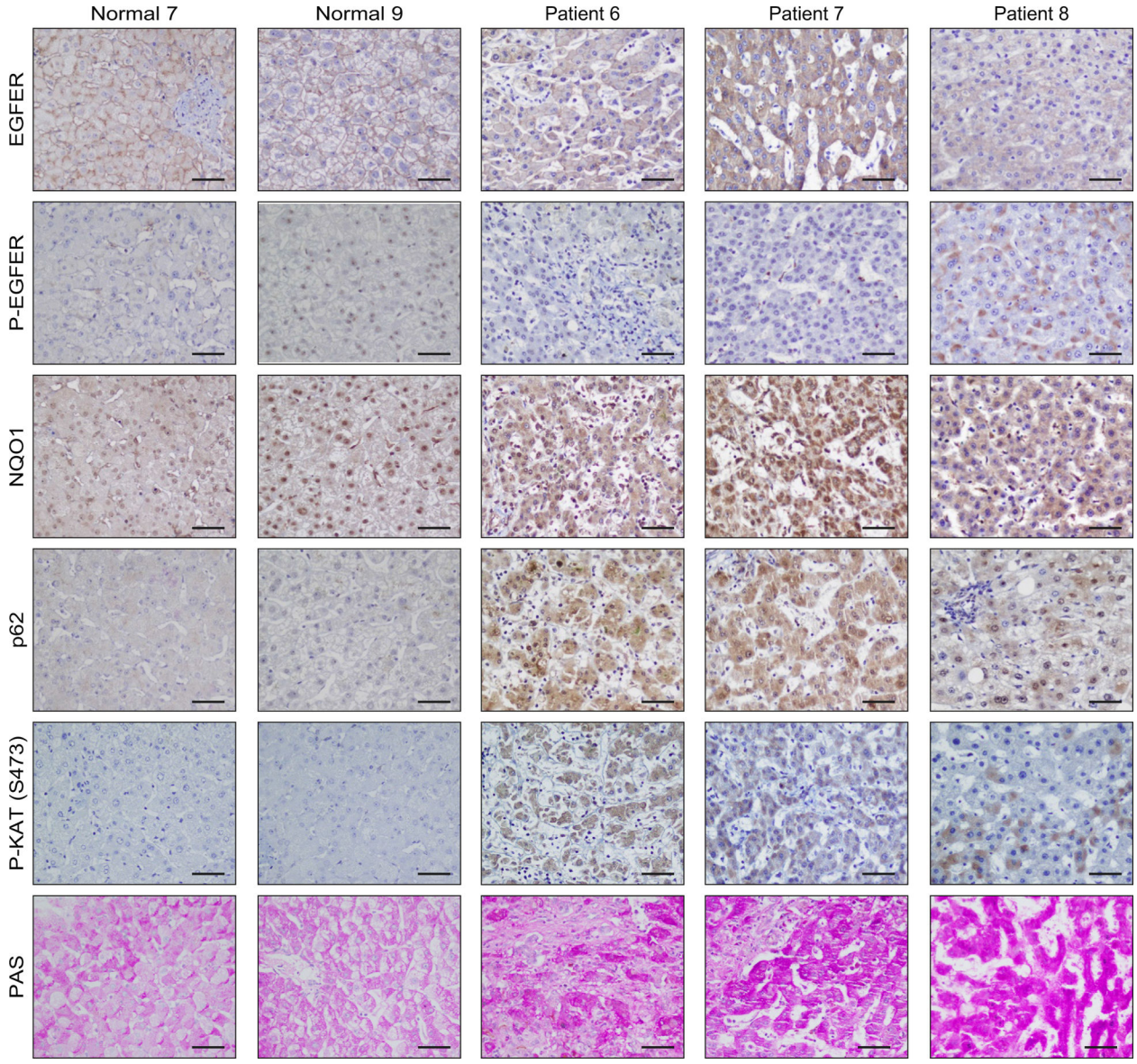


Fig. 8. The p62-NRF2-EGFR module is activated in human HSOS-related hepatomegaly.

Periodic acid-Schiff and immunohistochemistry staining of normal liver and HSOS hepatomegaly specimens with antibodies to EGFR, P-EGFR (Tyr1068), NQO1, p62 and P-AKT(Ser 473). Scale bars: 50 μm. n = 10. HSOS, hepatic sinusoidal obstruction syndrome.