

Manuscript Number: JHEPAT-D-16-01414R1

Title: Autotaxin-lysophosphatidic acid receptor signalling regulates hepatitis C virus replication

Article Type: Original Article

Keywords: hypoxia, autotaxin, lipid signalling, hepatitis C virus

Corresponding Author: Professor Jane A. McKeating, B.Sc., Ph.D.

Corresponding Author's Institution: University of Birmingham

First Author: Michelle J Farquhar, B.Sc., Ph.D.

Order of Authors: Michelle J Farquhar, B.Sc., Ph.D.; Isla S Humphreys, B.Sc., Ph.D.; Simon A Rudge, B.Sc., Ph. D.; Garrick K Wilson, B.Sc., Ph. D.; Bishnupriya Bhattacharya, B. Sc., Ph. D.; Maria Ciaccia, B.Sc., Ph.D.; Ke Hu, B.Sc.; Qifeng Zhang, B. Sc., Ph. D.; Laurent Mailly, B. Sc., Ph. D.; Gary M Reynolds, B. Sc., Ph. D.; Margaret Ashcroft, B.Sc., Ph.D.; Peter Balfe, Ph. D.; Thomas F Baumert, M. B. Ch. B., Ph. D.; Stephanie Roessler, B. Sc., Ph. D.; Michael J Wakelam, B. Sc., Ph. D.; Jane A McKeating, B. Sc., Ph. D.

Abstract: Background and aims: Chronic hepatitis C is a global health problem with an estimated 170 million HCV infected individuals at risk of progressive liver disease and hepatocellular carcinoma (HCC). Autotaxin (ATX) is a phospholipase with diverse roles in physiological and pathological processes including inflammation and oncogenesis. Clinical studies have reported increased ATX expression in chronic hepatitis C, however, the pathways regulating ATX and its role in the viral life cycle are not well understood. Methods: In vitro hepatocyte and ex vivo liver culture systems along with chimeric humanized liver mice and HCC tissue enabled us to assess the interplay between ATX and the HCV life cycle. Results: HCV infection increased hepatocellular ATX RNA and protein expression. HCV infection stabilizes hypoxia inducible factors (HIFs) and we investigated a role for these transcription factors to regulate ATX. In vitro studies show that low oxygen increases hepatocellular ATX expression and transcriptome analysis showed a positive correlation between ATX mRNA levels and hypoxia gene score in HCC tumor tissue associated with HCV and other aetiologies. Importantly, inhibiting ATX-lysophosphatidic acid (LPA) signalling reduced HCV replication, demonstrating a positive role for this phospholipase in the viral life cycle. LPA activates phosphoinositide-3-kinase that stabilizes HIF-1 α and inhibiting the HIF-signalling pathway abrogates the pro-viral activity of LPA. Conclusions: Our data support a model where HCV infection increases ATX expression that supports viral replication and HCC progression.

Response to Reviewers: A file containing the responses has been uploaded as a PDF file.



**THE UNIVERSITY
OF BIRMINGHAM**

Centre for Human Virology
MRC Centre for Immune Regulation
NIHR Centre for Liver Disease
The Medical School
University of Birmingham
Edgbaston
Birmingham B15 2TT
United Kingdom

Professor of Molecular Virology
Tel: 0121 414 8173
Email: j.a.mckeating@bham.ac.uk

Journal of Hepatology Editors

8th Dec 2016

Resubmission of manuscript JHEPAT-D-16-01414R 'Autotaxin-lysophosphatidic acid receptor signalling regulates hepatitis C virus replication'

Dear Editors

We are pleased to submit our revised manuscript on the role of autotaxin in the hepatitis C virus lifecycle. We'd like to take this opportunity to thank the reviewers for their constructive comments and provide a point-by-point response in the attached document. We believe the manuscript has been improved by these revisions and we look forward to a favourable response.

With my best wishes,

A handwritten signature in blue ink, reading 'J. McKeating', with a long horizontal flourish extending to the right.

Jane A McKeating.

Professor of Molecular Virology.

Responses to reviewers comments:

Reviewer #1: In this manuscript Farquhar and colleagues investigate the role of hypoxia in the regulation of autotaxin expression - which the authors describe as a novel pathway to modulate hepatitis C virus replication and HCC development. This is an interesting study, that in most of the parts is well controlled. Still, I believe that the paper needs several additional experiments and controls. Several molecular aspects of action are not clear and should be worked on.

Title: It is not clear where the authors show modulation of hepatocellular carcinoma. Thus, in case not clearly shown "hepatocellular carcinogenesis" should be omitted in the title. This can be and is discussed in the text but in case no data are shown it should not be put in the title. Response: We have edited the title to remove HCC pathogenesis. However, we have also included some new data in Fig.3c showing an association between high ATX RNA expression in HBV-associated HCC progression.

Figure 1a - It is not clear what is demonstrated in the two ATX blots in panel a. Why are there two bands in the cell vs. extracellular? Other loading control than beta-actin? The induction of ATX is impressive - loading control for the second ATX blot? kDa are missing! Response: ATX is a glycoprotein and the multiple bands in the cellular fraction represent glycosylated and non-glycosylated forms, whereas secreted ATX runs as a single band representing the glycosylated protein.

Fig.1b - The trend in PHH is visible - so convincing for me - maybe one could increase the n of PHH samples so that this trend is seen in 5 PHH samples altogether? Response: The chimeric SCID-upA mice are difficult to breed and our inclusion of data from several animals transplanted with PHH from three donors exceeds sample numbers included in many published reports using this model. Given these difficulties and the statistical significance of our results with three donors we believe the conclusions from these studies are valid.

Fig.1c - Scale bar is missing - special staining? Controls to show specificity? Cell of expression - is it the virus-infected cells - the neighbouring cells or both? Response: The revised figure includes irrelevant isotype stained liver sections and we have noted the x400 magnification in the figure legend. Since ATX is a secreted protein that can be taken up by diverse cell types we have revised the text in the manuscript to reflect this and to interpret the stains with care. It is notoriously difficult to visualize HCV antigen expressing hepatocytes in liver tissue from the chimeric mouse model or in infected patients and so it is beyond the scope of the current study to co-stain liver sections for ATX and viral encoded antigens or RNA.

Fig.1d - More Huh-7 cells to be shown (at least 3?). Response: We have replotted graph to show additional biological replicates.

Fig.1e and f - Link to different oxygenation? HIF expression? Histology to show a representative image? Response: The impact of local oxygen tension on the HCC microenvironment is an important research question that may be best answered using oxygen sensitive imaging techniques that are beyond the scope of this project. Given the difficulties stated above in interpreting anti-ATX stained biopsies we have deferred from staining the HCC tissues.

Fig.2: How does ATX expression develop under p_{HIF1}alpha by WB in Figure 2a? Response: Transient over-expression of HIF1a under normoxic conditions alters the balance between genesis: degradation, and promotes HIF-transcriptional responses as seen with VEGF mRNA levels.

Fig. 3a - How does HA130 treatment affect ATX expression? Response: HA130 is an antagonist of ATX enzymic activity and has no observed effect on ATX protein levels in our study or in the published literature.

Fig.3d - These experiments are very nice. However, the quality of the WB should be increased. It is not quite clear - in general all WBs lack kDa indication. Response: We have included the kDa markers in the revised figures.

Fig.4a - It is not clear how the different doses of LPA change cell integrity (e.g. proliferation, cell survival, expression of markers that support virus replication; this should be tested). Response: LPA had no discernable effect on hepatocyte morphology and promoted cellular proliferation, justifying our experiment (Fig.4f) to evaluate the effect of HA130 on HCV replication in growth arrested cells. These data suggest that the anti-viral activity of HA130 is independent of the proliferative status of the target cell.

Fig.4b - These data are convincing and very interesting. Is it possible to dissect LPA1 and 3?
Response: We have included LPA receptor RNA expression data in the revised Ms but further dissection of receptors would require shRNA or CRISPR and is beyond the scope of the current study.

Fig 5c: What additional effect does the high dose of HA130 (100 nm) have - are the effects directly attributable to suppression of ATX enzymatic activity? (Cell integrity? Apoptosis, survival, changes in the replication machinery?)
Response: no effects, ATX inhibitors have been safely used in clinical trials.

If the mechanism of HA130 is directly on ATX function - what are the downstream players that are changed.
Response: Fig.7 of the revised manuscript shows a role for LPA to activate PI3K and stabilize HIF-1a and we believe this signaling pathway promotes HCV RNA replication. We have provided a cartoon of the ATX-LPA signaling pathway in the HCV replicative life cycle (Fig.7f).

Could the authors investigate to some degree the underlying mechanisms of ATX inhibition and reduction in viral replication? To get some more insight on this would be important! It is not clear how this works.
Response: Please see our response above and provision of ATX-LPA signaling pathway in Fig.7f.

Fig.6b: The Akt phosphorylation is interesting indeed - is this something that is reproducible in other cells or PHH? Or is this specific to Huh-7 cells?
Response: LPA activates PI3K and thus AKT in a range of cell types – please see our earlier report - Jethwa SA, Leah EJ, Zhang Q, Bright NA, Oxley D, Bootman MD, Rudge SA, Wakelam MJO. (2016) "Exosomes bind autotaxin and act as a physiological delivery mechanism to stimulate LPA receptor signalling in cells." J Cell Sci. 129, 3948-3957.

In the context of LPA-activated PI3-kinase signalling in stabilizing HIF1a and HCV replication it would be important to show this in other cell systems.
Response: Several citations are provided in the manuscript that reference LPA stabilizing HIF1a in other cell systems.

On the whole this is an interesting paper - the title I believe is partially misleading as no real data on hepatocellular carcinogenesis are presented. So this should be omitted - I agree that the LPA signalling pathway might be an interesting target but this has not been shown in regards to HCC development. Also I believe the paper ends fast, but mechanistically several things are not clear.
Response: We have revised the title of the paper to exclude hepatocellular carcinogenesis. In addition we include new data in Fig.3c to ascertain the relationship between ATX RNA levels and HCC pathogenesis and believe this strengthens our study.

Reviewer #2: In their current manuscript, the authors investigate the regulation of autotaxin (ATX) and LPA signaling in the contexts of HCV infection and hypoxia. They describe, for the first time to my knowledge, that HCV infection upregulates ATX expression on both, transcriptional and protein levels, which in turn leads to the generation of the messenger molecule LPA. The authors furthermore show that ATX is also upregulated by low oxygen conditions (hypoxia), most probably through upregulation of the transcription factor HIF1a. Vice versa, they also describe upregulation of HIF1a by ATX / autocrine LPA signaling through the PI3K/AKT pathway. Lastly, they confirm their previous finding that inhibition/knockdown of HIF1a substantially impacts on HCV replication.

LPA signaling has been implicated with numerous malignancies, and ATX / LPA signaling are getting into the focus of researchers as anti-cancer targets (e.g. PMID: 25704398). Therefore, the authors' current finding of HCV-induced upregulation of ATX/LPA signaling is very interesting and proposes this process as a potential mechanism of carcinogenesis or at least as a tumor promoting factor in hepatitis C.

The manuscript is written and presented very clearly, concisely and comprehensibly. Data is presented in a clear and meaningful way, with almost all controls included, and statistical significance indicated where possible and necessary. For the largest part, the observed effects are unambiguous and the authors' conclusions are justified.

While reading the manuscript was pleasant and following the experiments was straightforward, the paper left one regrettably big question unanswered: the chicken and egg dilemma. The authors and a more or less ample set of published studies show many co-regulations and interdependences: HCV induces ATX; ATX produces LPA, which induces HIF1a; HIF1a again induces ATX; hypoxia induces HIF1a AND ATX; HIF1a and LPA promote HCV replication and

suppression of each also suppresses HCV.

Each effect alone is convincing, but together they leave the reader dizzy with no clear understanding of what is actually going on. In fact, it seems as if the authors themselves are not exactly sure, where they should be heading. The title of their story is "hypoxia regulates ATX expression", but Figure 1 first shows that HCV infection regulates ATX expression (which is interesting and important!) and secondly that hypoxia can do the same independent of the virus. For the whole rest of the story, hypoxia is unimportant and everything is centered around HCV (which does it all even under normoxia).

Response: We have included two new pieces of data and revised text to address this reviewer's comments and provided a cartoon to describe our model (Fig.7f).

- (1) Time course of VEGF and ATX expression following HCV infection – showing HIF target gene precedes ATX upregulation.
- (2) The HIF pathway inhibitor NSC reduces the pro-viral activity of LPA, supporting our model that LPA primed HCV infection is HIF-dependent.

Does HCV lead (as one would expect from the strong ATX upregulation) to an increase in LPA production, and if so, of which subtype? Might this suffice to induce HIF1a? **Response:** The requirement on safety grounds to 'heat inactivate infectious HCV' prior to mass spec analysis has distorted our LPA recovery and provided uncontrollable variable data. Despite our best efforts we have been unable to perform this experiment and we state this in the revised manuscript.

This leads to this question: is HIF1a still upregulated / stabilized by HCV if treated with LPAR-antagonist? If not, this would be a strong case for indirect HIF1a upregulation by HCV in contrast to direct stabilization. **Response:** Please see above and the new data presented in Fig.7b.

These are all just suggestions, which might or might not make sense. However, I do have the strong feeling that- despite the high quality of the science as it is currently already- it would require addressing such more causal / mechanistic aspects to make the manuscript ready for publication; this does not require a massive amount of additional experimentation. **Response:** We thank this reviewer for their excellent suggestions to improve the clarity of our manuscript and we believe our additional data along with text edits have addressed their concerns.

Beyond these more general issues, there are a few more technical points I would like to raise and propose the authors to modify in a potential revision:

Albeit difficult, I would very much suggest using a second, constitutively secreted protein as a control for the extracellular ATX Western blots. Would albumin work? Or some cytokine? If not, at least one control experiment showing HCV and hypoxia mediated ATX upregulation has to be performed with cells artificially secreting a control protein, e.g. Gaussia luciferase. Otherwise, controlling for cell numbers etc. is very difficult. **Response:** We controlled for cell numbers in all experiments and have stated this in the revised text.

Are really no other LPARs capable of mediating the observed effect? It seems like a luck shot, that the one and only tested inhibitor does the whole job. LPAR5 would of course be a hot candidate as it is expressed in liver (in contrast to LPAR1 and 2). Lacking an inhibitor, this would require RNAi, though. **Response:** We provide transcript data in Fig.5c to show that Huh-7 cells and PHHs express all LPARs, with the exception of LPAR4. Further studies to explore the LPAR dependency of LPA signaling in the HCV life cycle would require silencing of all six receptors and given the limited availability of validated antibodies this is beyond the scope of the current study.

Fig.4A: This experiment should better be done in DMSO "differentiated" cells. A 100-fold difference in LPA concentration yields only a less than 2.5-fold increase in HCV replication - may this be the pro-proliferative effect of LPA? **Response:** Data not provided in current Ms shows that DMSO 'differentiated' cells show a 100-fold reduced permissivity to support replication and we think this may be due to their blunted HIF response, this is the subject of an independent manuscript.

1. Fig.1 C should get a label ("ATX").

2. Fig. 1 E: I do not understand the Y-scale. What is a relative expression of -2?

3. All y-axes labels "Relative ATX expression" should make clear that mRNA is measured

4. Fig. 6D: labels misleading; make clearer that all four samples are LPA treated and are normalized to the LPA untreated, inhibitor untreated sample, which is not shown (or show it!).

Response: we have revised figures and text to respond to all of these points.

Reviewer #3: The authors demonstrate that autotaxin (ATX) has a positive effect on the HCV replication and provide evidence indicating that ATX expression is up-regulated under low oxygen conditions in a HIF-dependent manner. This was based on the use of in vitro hepatocyte cultures, chimeric humanized liver mice, HCC tissue and ex vivo liver culture systems. Furthermore, they claim that HCV infection increases hepatocellular ATX expression via stabilizing hypoxia inducible factor (HIF). Based on these data they propose a model suggesting that "HCV infection may increase hepatocellular ATX expression via HIF1 activation and that increases viral replication and establishes a paracrine LPA-signalling environment that drives fibrosis, cirrhosis and HCC pathogenesis"

The following points should be considered:

*The authors provide evidence indicating that low oxygen conditions or HCV infection independently positively regulate ATX expression. Provided that liver microenvironment is hypoxic, I recommend to test ATX expression in HCV-infected cells under low oxygen conditions and, should this be the case, to test the role of HIF. **Response:** Since low oxygen potentiates HCV infection (manuscript in preparation) and increases both the frequency of viral antigen expressing cells and their viral RNA burden – it would be difficult to discriminate the additive effects of these two cellular stresses on ATX expression. Given the anti-viral effects of siRNA HIF-1a and the complexities of discriminating between low oxygen and HCV infection induced changes in ATX expression we would defer from including this experiment in our manuscript.*

*Vassilaki et al. JVI 2013 reported that low oxygen conditions stimulate HCV replication in cultured cells and provided data that associate this phenomenon with HIF-independent changes of the cellular bioenergetics under low oxygen. Authors may wish to discuss this publication accordingly. **Response:** We have cited and discussed this work in our revised manuscript.*

*Page 4, lines 5-9: "Human hepatocytes in the transplanted mice expressed comparable levels of ATX mRNA to Huh-7 cells, however, following isolation and short-term propagation the PHHs showed significantly lower levels of ATX mRNA (Fig. 1d) that may reflect their de-differentiation in vitro". Again, I recommend to perform these experiment under low oxygen conditions. Furthermore, authors may show experimental evidence for the proposed de-differentiation. **Response:** We have revised our manuscript to state that PHHs show reduced levels of hepatocyte specific differentiation markers compared to human liver tissue, suggesting a de-differentiation of the cells in vitro. We would be very interested to investigate the effect of low oxygen on human hepatocellular ATX expression and this would be most elegantly assessed using the chimeric human-mouse liver tissue. Unfortunately since the mice are housed in our collaborator Prof Baumerts laboratory in Strasbourg this experiment has not been possible at the present time.*

*Fig 2b: Data from statistical analysis are presented only for the ATX mRNA. Present/discuss statistical analysis for the mRNA levels of the control VEGF or GLUT1 genes. **Response:** We provides stats for these data in the revised manuscript.*

Autotaxin-lysophosphatidic acid receptor signalling regulates hepatitis C virus replication

Michelle J Farquhar^{1*}, Isla S Humphreys^{1*}, Simon A Rudge^{2*}, Garrick K Wilson¹,
Bishnupriya Bhattacharya¹, Maria Ciaccia², Ke Hu¹, Qifeng Zhang², Laurent Maily³,
Gary M Reynolds⁴, Margaret Aschcroft⁵, Peter Balfe¹, Thomas F Baumert³,
Stephanie Roessler⁶, Michael JO Wakelam^{2**} and Jane A McKeating^{1**†}

1. Viral Hepatitis Laboratory, Centre for Human Virology, University of Birmingham, UK.
2. The Babraham Institute, Cambridge, UK.
3. INSERM U1110, University of Strasbourg, 3 Rue Koeberlé, F-67000 Strasbourg, France.
4. NIHR Liver Biomedical Research Unit, University of Birmingham, Birmingham, UK.
5. Cambridge Biomedical Campus, University of Cambridge, Cambridge
6. Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany.

* Joint first and ** senior authorship.

† Corresponding author Jane A. McKeating, contact information: j.a.mckeating@bham.ac.uk

Tel: (44) 121 414 8173, fax: (44) 121 414 3599

Conflicts of interest: There are no conflict of interest to disclose for any of the authors.

Abbreviations: Alcoholic liver disease (ALD); Autotaxin (ATX); Direct acting antivirals (DAAs); Epstein Barr virus (EBV); hepatitis B virus (HBV); hepatocellular carcinoma (HCC); hepatitis C virus (HCV); HCV pseudoparticle (HCVpp); Hypoxia inducible factor (HIF); lysophosphatidic acid (LPA); lysophosphatidylcholine (LPC); normal liver (NL); primary human hepatocyte (PHH); relative light units (RLU).

Key words: Autotaxin, lipid signalling, hepatitis C virus, hypoxia.

Author involvement: MF, IH, GKW, SR and MC designed and performed the work; KH, BP, QZ and PB performed experiments; SR, LM, MA and TB provided reagents; MJF, SR, JAM and MJOW designed the study and wrote the manuscript.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Abstract:

Background and aims: Chronic hepatitis C is a global health problem with an estimated 170 million HCV infected individuals at risk of progressive liver disease and hepatocellular carcinoma (HCC). Autotaxin (ATX) is a phospholipase with diverse roles in physiological and pathological processes including inflammation and oncogenesis. Clinical studies have reported increased ATX expression in chronic hepatitis C, however, the pathways regulating ATX and its role in the viral life cycle are not well understood. **Methods:** In vitro hepatocyte and ex vivo liver culture systems along with chimeric humanized liver mice and HCC tissue enabled us to assess the interplay between ATX and the HCV life cycle. **Results:** HCV infection increased hepatocellular ATX RNA and protein expression. HCV infection stabilizes hypoxia inducible factors (HIFs) and we investigated a role for these transcription factors to regulate ATX. In vitro studies show that low oxygen increases hepatocellular ATX expression and transcriptome analysis showed a positive correlation between ATX mRNA levels and hypoxia gene score in HCC tumor tissue associated with HCV and other aetiologies. Importantly, inhibiting ATX-lysophosphatidic acid (LPA) signalling reduced HCV replication, demonstrating a positive role for this phospholipase in the viral life cycle. LPA activates phosphoinositide-3-kinase that stabilizes HIF-1 α and inhibiting the HIF-signalling pathway abrogates the pro-viral activity of LPA. **Conclusions:** Our data support a model where HCV infection increases ATX expression that supports viral replication and HCC progression.

1 **Introduction:** Chronic viral hepatitis is a global health problem with at least 170 million
2 hepatitis C virus (HCV) infected individuals at risk of developing liver disease that can
3 progress to hepatocellular carcinoma (HCC). The recent availability of direct acting anti-viral
4 agents can eliminate HCV in up to 90% of patients[1]. However, the high cost of these drugs
5 along with reports of viral genotype resistance, may limit their wide-spread use. Importantly,
6 patients with liver cirrhosis cured of HCV may remain at risk of developing HCC, highlighting
7 the need to understand host pathways playing a role in HCC development[2, 3].

8 Autotaxin (ATX) is a member of the ectonucleotide pyrophosphatase/phosphodiesterase
9 family of proteins that was identified as a motility-stimulating factor secreted from
10 melanoma cells[4]. ATX hydrolyzes lysophosphatidylcholine (LPC) to lysophosphatidic acid
11 (LPA), a growth factor that activates and signals via a family of six G-protein coupled LPA
12 receptors (LPA₁₋₆). The ATX-LPA signalling axis has been reported to play a tumorigenic role
13 in a wide number of cancers and is a candidate for therapeutic intervention[5]. Several
14 studies have reported increased ATX and LPA levels in the plasma of HCV infected subjects
15 that associates with liver fibrosis staging[6-9]. A recent prospective study showed that
16 serum ATX predicts the severity of liver cirrhosis and prognosis of cirrhotic patients[10].
17 Mazzocca and colleagues reported that HCC secreted LPA increases the trans-differentiation
18 of peritumoral fibroblasts to carcinoma associated fibroblasts that are considered to play a
19 role in tumour proliferation and metastasis[11].

20 ATX is expressed in many tissues and the mechanisms accounting for increased serum
21 phospholipase activity in chronic hepatitis C and the impact on viral replication are not
22 understood. We show that HCV infection of hepatocyte-derived cells or mice with humanized
23 chimeric livers increases ATX mRNA and protein expression. Infection stabilizes hypoxia
24 inducible factor-1 α (HIF-1 α)[12, 13] and we show that low oxygen increases ATX transcripts
25 in human liver slices, suggesting a pathway for HCV to regulate ATX. We demonstrate a
26 positive association between ATX and hypoxia related gene expression in viral and non-viral
27 HCC, providing an explanation for elevated ATX expression in tumors that are frequently
28 hypoxic. Finally, we demonstrate that ATX-LPA signalling regulates HCV RNA replication via
29 a phosphoinositide 3 kinase (PI3K) dependent pathway, demonstrating a role for
30 lysophospholipids in viral infection. Our data support a model where HCV infection increases
31 hepatocellular ATX expression that promotes viral replication and establishes a paracrine
32 LPA-signalling environment leading to fibrosis and HCC pathogenesis.

33 Results

34 **HCV infection and low oxygen induce autotaxin expression.** To ascertain whether HCV
35 infection directly regulates ATX expression we selected Huh-7 hepatocyte-derived cells as a
36 permissive target cell that supports HCV replication. *In vitro* tissue culture protocols routinely
37 use media containing bovine serum that contains high levels of ATX that can catalyse LPC-
38 LPA conversion. We therefore performed all experiments under serum-free conditions to limit
39 the confounding effects of *de novo* generated bovine LPA. Huh-7 cells express ATX and the
40 majority of protein is detected in the extracellular media (**Fig.1a**). HCV (strain J6/JFH)
41 infection induced a significant increase in ATX mRNA and protein expression (**Fig.1a**).
42 Experiments to assess the effect of HCV infection on primary human hepatocyte (PHH) ATX
43 expression were inconclusive due to their low permissivity under serum-free conditions
44 required to interrogate human ATX function.

45 The uPA-SCID human liver chimeric mouse supports HCV infection[14] and enables us to
46 study the effect of viral infection on hepatocellular ATX expression *in vivo*. Since ATX is likely

1 to be expressed by multiple cell types in the human liver this murine model provides a
2 unique opportunity to ascertain whether hepatocytes express ATX. All mice engrafted with
3 human hepatocytes express human ATX and infection increased ATX mRNA levels,
4 independent of the hepatocyte donor (**Fig.1b**). In non-transplanted mice we failed to detect
5 ATX expression illustrating both the specificity of the primers for human ATX and
6 demonstrating that HCV-dependent modulation of ATX is of human hepatocyte origin. It is
7 interesting to note that hepatocytes in the transplanted mice express comparable levels of
8 ATX mRNA to Huh-7 cells, however, following isolation and short-term propagation PHHs had
9 lower ATX mRNA levels (**Fig.1c**). We noted a 10-20 fold reduction in mRNA levels of
10 hepatocyte specific markers (Albumin, CYP3A4 and HNF4a) in cultured PHHs during the first
11 48h of culture, most likely reflecting their de-differentiation. Immunohistochemical staining
12 of the chimeric murine-human livers showed hepatocytes expressing ATX in the HCV infected
13 animals (**Fig.1d**), however, given the secreted nature of this protein we should interpret
14 these data with care with respect to the cellular source of the stained ATX. Next, we sought
15 to analyse ATX RNA levels in HCC from patients diagnosed with HCV, hepatitis B virus (HBV)
16 and alcoholic liver disease (ALD) [15, 16]. Transcriptomic analysis showed a significant
17 increase of ATX in HCV-associated HCC but also in subjects with HBV and ALD compared to
18 normal liver (NL) (**Fig.1e**). A pairwise analysis of tumour and non-tumour tissue from a
19 cohort of 233 Chinese patients with HBV-associated HCC showed that ATX is significantly
20 upregulated in tumour compared to non-tumour tissue (**Fig.1f**).

21
22
23
24 We observed increased ATX mRNA levels in HCV-infected mice, HCC tumours and Huh-7 cells
25 suggesting that infection perturbs ATX at the transcriptional level. Since HCV can stabilize
26 hypoxia inducible factor 1 α (HIF-1 α) [12, 13] we investigated a potential role for this
27 transcription factor to regulate ATX. Under normoxia HIF- α subunits are rapidly targeted for
28 proteosomal degradation by prolyl hydroxylases, however, under low oxygen conditions
29 these hydroxylases are inactivated resulting in stable HIF expression. Huh-7 cells were
30 cultured under normoxic (20%O₂) or hypoxic (1%O₂) conditions for 24h and ATX mRNA
31 levels along with HIF-target genes VEGF and GLUT1 were quantified. We confirmed HIF-1 α
32 expression by Western blotting and observed a significant increase in ATX, VEGF and GLUT1
33 mRNA and secreted ATX from Huh-7 cells cultured under low oxygen (**Fig.2a**).

34
35
36
37
38 To analyse a role for HIF-1 α in regulating ATX expression, Huh-7 cells were transfected with
39 a plasmid encoding HIF-1 α to express the transcription factor under normoxic conditions. We
40 demonstrated a modest level of HIF-1 α expression and increased ATX and VEGF mRNA levels
41 (**Fig.2a**), suggesting a role for HIF in regulating ATX transcription. Further studies
42 investigated the effect of low oxygen on ATX expression in human liver slices. Liver slices
43 from 5 independent donors cultured under normoxic or hypoxic conditions for 24h showed a
44 significant increase in ATX, VEGF and GLUT1 mRNA levels (**Fig.2b**), demonstrating a role for
45 low oxygen in regulating ATX expression in human liver tissue. To ascertain whether low
46 oxygen regulates ATX promoter activity we cloned the published promoter region[7] into a
47 reporter plasmid and quantified luciferase activity in Huh-7 cells cultured under normoxic or
48 hypoxic conditions for 24h. The ATX promoter plasmid showed an approximate 2-log
49 increase in luciferase activity compared to vector alone, however, this was not increased by
50 culturing the cells under hypoxic conditions (**Fig.2c**). As a control Huh-7 expressing a
51 hypoxia responsive element (HRE) reporter, showed a 200-fold increase in luciferase activity
52 (**Fig.2c**). The negligible effect of low oxygen on ATX promoter activity is consistent with the
53 absence of HREs in this region and the lack of HIF-1 α binding to this region in HepG2
54 hepatoma cells by ChIP-SEQ (David Mole, personal communication). Collectively, these data
55 support an indirect role for HIF-1 α to regulate ATX transcription.

1 To understand whether HCV upregulates ATX expression via a HIF-dependent pathway we
2 quantified ATX and VEGF mRNA levels over time and only observed a significant increase in
3 ATX mRNA levels 48h post infection (**Fig.2d**). In contrast, we observed increased VEGF
4 mRNA levels after 24h and they remained elevated for the duration of the experiment
5 (**Fig.2d**). Assuming similar half-lives for these two RNA species these data support a model
6 where HCV stabilized HIF-transcriptional activity precedes ATX upregulation. To validate
7 these conclusions we siRNA silenced HIF-1 α in Huh-7 cells to assess its role in viral
8 regulation of ATX and Western blotting confirmed effective silencing under low oxygen
9 conditions (**Fig.2e**). However we failed to infect these cells, suggesting a role for HIF-1 α in
10 the HCV life cycle as previously reported[13] but preventing confirmation of this pathway in
11 viral regulation of ATX.

12
13 To extend our observations that hypoxia regulates ATX we studied the relationship between
14 ATX and hypoxia gene transcript levels in HCC. van Malenstein *et al* identified a seven gene
15 'hypoxia signature' that was elevated in HCC[17]. We investigated the expression of these
16 genes in our HCC cohorts. We noted a significant deregulation of five out of seven hypoxia-
17 regulated genes and a significant increase in the hypoxia score in HCC associated with all
18 aetiologies compared to NL (**Supplementary Fig.1**). Since the time of processing liver
19 samples can vary and may result in oxygen deprivation and HIF expression, we selected to
20 study the HBV cohort where tumour and adjacent non-tumour tissue was available, enabling
21 us to limit sampling artefacts and to conduct pairwise comparisons. We observed an
22 increased hypoxia gene score in HCC compared to matched non-tumour tissues (**Fig.3a**).
23 Furthermore, we noted a positive correlation between ATX mRNA and the hypoxia gene
24 signature (**Fig.3b**). Due to the reported association of hypoxia and patient survival, we
25 asked whether ATX gene expression associates with patient outcome[17]. Patients were
26 classified in two groups based on the median difference of ATX expression between tumour
27 and non-tumour tissues. Kaplan-Meier survival curves revealed that patients with high ATX
28 expression had shorter survival times compared to low expression, although this difference
29 was not statistically significant (log-rank p-value) (**Fig.3c**). Thus, we could confirm that the
30 Hypoxia score is increased in HCC tumour tissue compared to adjacent non-tumour tissue
31 and ATX mRNA expression positively correlates with the Hypoxia gene signature supporting a
32 role for hypoxia to regulate ATX-LPA signalling in HCC development.

33
34 **Autotaxin-LPA signalling axis in HCV infection.** To investigate a role for ATX in the HCV
35 life cycle we treated Huh-7 cells with HA130, a selective inhibitor of ATX enzymatic
36 activity[18]. HA130 had no effect on Huh-7 viability but significantly reduced LPA levels
37 (**Fig.4a**). LPA is not a single entity and mass spectrometric analysis shows that Huh-7
38 generate several species of differing acyl chain lengths and degrees of saturation, with 16:0
39 and 18:1 being the most abundant. Statistical analysis of the results from five independent
40 experiments showed a significant reduction in 18:1, 18:2, 20:4 and 22:6 LPA levels from
41 HA130 treated cells compared to the untreated control. In contrast, HA130 had no significant
42 effect on expression of LPA species 14:0, 16:0 or 18:0 (**Fig.4a**). We observed a dose-
43 dependent HA130 inhibition of HCV J6/JFH and SA13/JFH infection (**Fig.4b**). To confirm
44 these observations, Huh-7 cells were transduced with an shRNA targeting ATX or an
45 irrelevant control. RT-PCR and Western blotting confirmed a reduction in ATX expression and
46 HCV infection (**Fig.4c**). Since off-target effects are a common limitation of gene silencing, we
47 performed a rescue experiment where ATX-silenced cells were transfected to express wild
48 type (ATX_{wt}) or a catalytically inactive mutant (ATX_{T210A}). Exogenous expression of ATX_{wt} in
49 silenced cells restored infection to levels seen in irrelevant shRNA transduced cells, whereas
50 the ATX_{T210A} mutant had no effect (**Fig.4d**). Of note, expressing ATX_{wt} in shControl or non-
51 transduced Huh-7 cells increased the frequency of HCV infected cells, suggesting that ATX

1 levels may be limiting. We confirmed that silencing ATX ablated the anti-viral effect of HA130
2 and exogenous LPA restored HCV infection (**Fig.4e**). To determine whether HCV increased
3 ATX expression drives higher LPA generation we attempted to quantify LPA species in mock
4 and infected samples by mass spectrometry. Unfortunately, the protocol necessary to
5 inactivate the virus prior to analysis, heating the samples at 65°C for 10 minutes, caused a
6 2-3 fold increase in all molecular LPA species most likely explained by heat-induced LPC
7 breakdown. Alternative inactivation protocols involving addition of chloroform/methanol
8 mixtures to the samples were tested but were incompatible with the extraction procedure
9 used for LC-MS analysis.

10
11 LPA can regulate cellular proliferation and HA130 treated Huh-7 cells show an increased
12 doubling time from 17h to 23h. To assess whether the anti-viral effect of HA130 is
13 dependent on Huh-7 proliferative status we used DMSO-arrested and differentiated Huh-7
14 cells[19]. We confirmed that the Huh-7 were cell cycle-arrested and showed increased levels
15 of differentiation markers albumin, CYP3A4 and HNF4- α (**Fig.4f**). The differentiation protocol
16 had no effect on ATX mRNA levels and treating with HA130 reduced HCV infection to a level
17 comparable seen with non-differentiated cells (**Fig.4f**), demonstrating that viral inhibition is
18 not linked to the proliferative status of the target cell.

19
20
21
22 Since our experiments demonstrate a requirement for the lysophospholipase activity of ATX
23 in HCV infection, we investigated the effect of exogenous LPA (18:1) on Huh-7 permissivity
24 for HCV infection. LPA enhanced HCV infection in a dose-dependent manner (**Fig.5a**). To
25 investigate the receptor dependency of LPA-augmented HCV infection, cells were incubated
26 with LPA in the presence or absence of the LPA_{1/3} antagonist Ki16425[20]. Ki16425
27 abrogated the pro-viral activity of LPA for HCV infectivity (**Fig.5b**), demonstrating a role for
28 LPA₁ or LPA₃ in HCV infection. LPA binds and signals through a family of six LPA receptors
29 and Huh-7 cells express mRNA for each of the receptors with the exception of LPA₄ at
30 comparable levels to PHHs (**Fig.5c**).

31
32
33
34 **A role for autotaxin in the HCV lifecycle.** To assess whether ATX has a specific role in
35 HCV entry into hepatocytes, we used lentiviral pseudoparticles expressing HCV E1E2
36 glycoproteins to measure glycoprotein-receptor entry. We selected HCV strain H77 and 1A38
37 glycoproteins as they routinely provide the most infectious pseudoparticle stocks[21]. HA130
38 had no effect on HCVpp infection (**Fig.6a**), similar observations were made with viruses
39 expressing a range of HCV glycoproteins (data not shown). To investigate a role for ATX-LPA
40 signalling in HCV RNA replication we treated Huh-7 cells stably expressing a subgenomic HCV
41 replicon encoding a luciferase reporter (Luc2a-JFH) with HA130 or shATX and noted a
42 significant reduction in luciferase activity (**Fig.6b**). To assess whether HA130 treatment of
43 naïve Huh-7 cells could limit the initiation of HCV RNA replication, Huh-7 cells were
44 transfected with a full-length HCV RNA encoding a secreted gaussian luciferase (JC1GLuc) and
45 we noted a dose-dependent HA130 inhibition of replication (**Fig.6c**). These studies show that
46 inhibiting ATX reduced HCV RNA replication in stably transfected cells and in naïve cells
47 challenged with virus, demonstrating a role for the ATX-LPA signalling in the initiation and
48 maintenance of viral replication.

49
50
51
52
53
54 LPA was reported to stabilize HIF-1 α expression in ovarian and colon cancer cells[22] and we
55 investigated a role for HIF-1 α in LPA-dependent HCV infection. We show that LPA stabilized
56 HIF-1 α under normoxic conditions and increased HRE-transcriptional reporter activity that
57 was inhibited by Ki16425 (**Fig.7a**), demonstrating LPA_{1/3} dependent signalling. We previously
58 reported that the HIF-pathway inhibitor NSC-134754[23] reduced hepatocellular HIF-1 α
59 expression and HCV replication[13]. Titration of NSC-134754 identified a sub-saturating

1 concentration (25nM) that reduced HIF-transcriptional reporter activity with minimal effect
2 on HCV replication and demonstrated a role for the HIF-signalling pathway in the pro-viral
3 activity of LPA (**Fig.7b**). Previous studies reported a role for PI3K activation in LPA-
4 dependent stabilization of HIF-1 α [24] and we found that LPA induced AKT phosphorylation in
5 Huh-7 cells (**Fig.7c**). Pre-incubating the cells with the pan PI-3-kinase inhibitor wortmannin
6 (WM) or BYL-719, that selectively targets p110 α class IA PI-3-kinase, abrogated HIF-1 α
7 expression (**Fig.7d**). In contrast, LPA-stimulated HIF-1 α expression was insensitive to the
8 presence of p110 β class IA PI3K inhibitor TGX-221 (**Fig.7d**). To establish a role for the PI-3-
9 kinase pathway in LPA-stimulated HCV infection we assessed the ability of these inhibitors to
10 modulate infection. WM and BYL-719 treatments ablated the LPA enhancement of HCV
11 infection whereas TGX-221 had no effect (**Fig.7e**). In summary, these data demonstrate a
12 role for LPA-activated PI-3-kinase signalling in stabilizing HIF-1 α that regulates HCV
13 replication (**Fig.7f**).
14
15

16 **Discussion:** Our studies uncover a role for the ATX-LPA signalling axis to positively regulate
17 HCV RNA replication by activating PI3K and stabilizing HIF-1 α (**Fig.7f**). Inhibiting ATX
18 activity or LPA signalling reduced HCV replication, providing evidence for an autocrine LPA-
19 feedback loop to promote viral replication. PI3K signalling has been reported to positively
20 regulate HCV replication[25] and suppressing this pathway inhibits HCV replication [26, 27].
21 We previously reported that low oxygen stabilized HIF promotes HCV infection[13] and our
22 current study showing that silencing HIF-1 α limits HCV replication, suggests a role for this
23 pathway in LPA-induced infection. Vassilaki et al reported that low oxygen stimulated HCV
24 replication[28], however, the authors concluded that this phenotype was independent of HIF-
25 1 α or HIF-2 α that may reflect the use of different Huh-7 cell clones or partial HIF-silencing.
26 Our observation that LPA stabilized hepatocellular HIF-1 α and is consistent with reports
27 showing a role for LPA to 'rescue' mesenchymal stromal cells[29] or human CD34+ cells[30]
28 in ischemic disease and are most likely explained by its ability to activate HIF signalling.
29
30
31

32
33 We demonstrate a role for low oxygen to regulate ATX mRNA in hepatocyte-derived Huh-7
34 cells and human liver slices, consistent with reports of increased ATX expression in a variety
35 of tumours that are frequently hypoxic. Importantly, we show a positive association between
36 elevated ATX mRNA levels in HCC and the hypoxia gene score. Transient over-expression of
37 HIF-1 α in Huh-7 cells increases ATX mRNA, suggesting an activating role for this
38 transcription factor. However, low oxygen had a minimal effect on ATX promoter activity, in
39 agreement with the lack of HRE sites in this region and suggesting that enhancer regions
40 beyond the published 1.2kb promoter may bind HIFs or that low oxygen regulated factors
41 increase ATX mRNA half-life and/or protein stability. Wu and colleagues reported that TNF α
42 induced a modest 3-fold increase in ATX mRNA levels in HepG2 cells via nuclear factor kappa
43 beta activation[7]. However, we failed to see any evidence for TNF α modulation of ATX
44 promoter activity in Huh-7 cells or human liver slices, suggesting that this may be cell type
45 dependent.
46
47
48
49

50 ATX is expressed in many tissues, however the source of elevated ATX in the sera of chronic
51 hepatitis C patients is unknown. Our studies with chimeric liver uPA-SCID mice show that
52 hepatocytes express ATX and HCV infection induces its expression in the absence of any
53 inflammatory response. We confirmed increased ATX transcript levels in HCC tumour tissues
54 from subjects with HCV, HBV and ALD aetiologies, demonstrating that increased ATX
55 expression is not unique to HCV infection. Reports that HBV can stabilize HIF[31] and ALD is
56 associated with hepatic HIF expression[32] lend support to our model that HIFs regulate
57 hepatic ATX expression. In the healthy liver ATX is most likely removed from the circulation
58 by sinusoidal endothelial cells[33], however, during fibrosis phenotypic changes in the
59
60
61
62
63
64
65

1 sinusoidal endothelium[34] are likely to impair ATX clearance that may account for the
2 increased expression reported in the fibrotic liver. However, these morphological changes are
3 unlikely to account for the increase in ATX mRNA observed in this study. It is interesting to
4 note that Epstein Barr virus (EBV) infection of Hodgkin lymphoma cells induces ATX
5 expression that augments their proliferation and survival[35]. EBV is an oncogenic virus
6 associated with B-lymphoid and non-lymphoid malignancies that is known to stabilize HIF-
7 1α [36], suggesting a common pathway for viruses to activate the ATX-LPA signalling axis.

8 LPA is not a single entity and exists in several forms with differing acyl chain lengths and
9 degrees of saturation that interact with specific LPA receptors and regulate physiological
10 responses. For example 18:1 LPA activates all receptors, whereas 20:4 LPA shows a higher
11 potency to activate LPA₃[37]. Huh-7 cells expressed a range of LPA molecular species and
12 HA130 showed differential effects on the genesis of some LPA species. These results
13 highlight potential differences in the role of LPA molecular species in the viral life cycle,
14 however, this variability may reflect differences in LPC substrate availability and/or lipid
15 phosphate phosphatases that may selectively degrade LPA species.

16 LPA signals through binding to a family of G-protein coupled receptors that can activate
17 signalling pathways including PI3 kinase and adenylyl cyclase to induce physiological changes
18 including cellular proliferation, anti-apoptosis and migration. Whilst LPA receptor over-
19 expression studies suggest that individual receptors can regulate physiological responses,
20 our understanding of tissue-specific LPA-signalling is limited. PHHs and Huh-7 express all of
21 the LPARs at the mRNA level with the exception of LPA₄ in Huh-7 cells. The ability of LPA_{1/3}
22 antagonist Ki16425[20] to limit HCV infection suggests a direct role for LPA₁ or LPA₃ in viral
23 replication. LPA signalling has been reported to drive chronic wound healing leading to
24 fibrosis and LPA modulators are in development for treating fibrosis[38]. A recent study
25 reported a role for LPA₆ in maintaining the proliferative capacity and tumorigenic phenotype
26 of HCC via the transcriptional activation of proto-oncogene *Pim-3*[39], highlight the value of
27 LPA receptor-targeted therapies for treating HCC.

28 HCC aetiology is multifactorial and the disease is often preceded by other conditions
29 including liver fibrosis and cirrhosis that are associated with HCV, HBV, alcoholic and non-
30 alcoholic hepatitis. The discovery of new therapeutic targets will require a greater
31 understanding of the pathogenic mechanisms underlying the tumorigenic process.
32 Intrahepatic HCC metastases are common and the tumour microenvironment is considered
33 to be pro-metastatic. Reports that elevated serum LPA associate with HCC tumour size and
34 patient survival[11], along with resistance to chemotherapy and radiation-induced cell
35 death[40], provide compelling evidence to consider the ATX-LPA axis as a therapeutic target
36 for treating HCC[41]. The embryonic lethality of ATX null mice[42-44] raised questions on
37 the suitability of ATX as a drug target. However, a recent report from Katsifa and colleagues
38 showing that inducible, ubiquitous genetic deletion of ATX in adult mice, and long-term
39 pharmacologic inhibition were well tolerated limits some of these concerns[45]. In summary,
40 we demonstrate a role for ATX-LPA signalling in the HCV lifecycle, highlighting potential new
41 targets for therapy and the prospect of stratifying therapies for treating viral-associated and
42 non-associated HCC.

1 **Acknowledgements:** We thank Takaji Wakita for HCV J6/JFH, Jens Bukh for HCV SA13/JFH
2 Charlies Rice for anti-NS5A mAb 9E10, Robert Thimme for Luc2a-JFH replicon cells and
3 Samantha Lissaeur for differentiation protocols. Statistical analysis on mass spectrometric
4 data reported in Fig.4a was performed by Dr. Anne Segonds-Pichon at the bioinformatics
5 facility at the Babraham Institute. Research in the McKeating laboratory was funded by the
6 MRC, NIHR Birmingham Liver BRU, EU FP7 PathCO and H2020 grant Hep-CAR. Research in
7 the Wakelam lab is supported by BBSRC and Hep-CAR. Stephanie Roessler was supported by
8 Hep-CAR, DFG grant RO4673, the Olympia-Morata Programme, a Brigitte-Schieben-Lange
9 Fellowship and a Heidelberg School of Oncology Fellowship.

10 **Figure legends.**

11 **Fig.1. HCV induces ATX expression.** (a) ATX expression in equal amounts of cellular
12 protein or extracellular media from mock or HCV J6/JFH infected Huh-7 cells at 48h post-
13 infection. Secreted ATX signals were measured by densitometry (annotated on Western
14 blots) and expressed relative to the intracellular pool or mock values. Cells were lysed for
15 total RNA preparation and ATX and GAPDH mRNA levels measured by real time PCR. (b) ATX
16 mRNA levels in mock (n=7) or HCV (n=6) infected uPA-SCID mice transplanted with primary
17 human hepatocytes (PHHs), where data is separated according to donor. Serum HCV RNA
18 levels at the time of sacrifice varied from 9,300-34,200 IU/mL. ATX mRNA levels are
19 expressed relative to uninfected cells (**p<0.01). (c) ATX mRNA levels in uPA-SCID human
20 liver chimeric mouse tissue (from 3 PHH donors), short-term cultured PHHs (4 donors) and
21 Huh-7 cells expressed relative to GAPDH (**p<0.01). (d) Representative
22 immunohistochemical ATX staining in uninfected and HCV infected uPA-SCID liver tissue
23 (x400 magnification). All data sets are representative of at least two independent
24 experiments. (e) ATX mRNA levels in HCC tumour tissue from patients with underlying HCV
25 (n=9), HBV (n=8), ALD (n=8), and normal healthy liver tissues (n=7) (*Mann-Whitney U
26 test p<0.05). (f) ATX mRNA levels in HBV-associated HCC tumour and paired non-tumour
27 tissue (***Wilcoxon p<0.001).

28 **Fig.2. Low oxygen regulates ATX expression.** (a) Huh-7 cells were cultured under 20%
29 or 1% oxygen for 24h and analysed for HIF-1 α expression; ATX, VEGF and Glut1 mRNA
30 levels and secreted ATX (**p<0.01). Huh-7 cells were transfected to express HIF-1 α and
31 48h later assessed for HIF-1 α expression, ATX and VEGF mRNA levels (*p<0.05: **p<0.01).
32 ATX protein signals were quantified by densitometry and hypoxic samples expressed relative
33 to normoxic ones. (b) Human liver slices from 5 independent donors were incubated under
34 20% or 1% oxygen for 24h and total RNA screened for ATX, VEGF and GLUT1 mRNA levels
35 (**p<0.001). All gene transcripts are expressed relative to GAPDH housekeeping gene. (c)
36 Huh-7 cells expressing ATX promoter-Luc or HRE-Luc were cultured under 20% or 1%
37 oxygen for 24h and luciferase activity measured (**p<0.01: ***p<0.001). All data sets are
38 representative of at least two independent experiments. (d) Temporal expression of ATX and
39 VEGF mRNA levels following HCV infection of Huh-7 cells. Infection was assessed by PCR
40 measurement of viral RNA copies at 12, 24 and 48h and were 1.7 x 10³, 8.5 x 10⁵ and 4.6 x
41 10⁷ RNA copies/10⁵ cells, respectively. (e) Huh-7 cells were transfected with siRNA targeting
42 HIF-1 α (siHIF) or control (siControl) for 48h, propagated under 1%O₂ for 24h to confirm HIF
43 silencing by Western blotting or infected with HCV SA13/JFH for 24h and infection assessed
44 by enumerating the frequency of NS5A expressing cells.

45 **Fig.3. Association of ATX and hypoxia gene score in HBV-HCC and impact on tumor**
46 **progression.** (a) Hypoxia seven-gene signature in HBV-associates HCC tumour and paired
47 non-tumour tissues (N=233, Wilcoxon p<0.001). (b) Correlation of relative ATX difference
48
49
50
51
52
53
54
55
56
57

1 between tumour and paired non-tumour tissues and hypoxia seven-gene signature in HBV-
2 associated HCC (Pearson correlation coefficient $r = 0.2788, p < 0.0001$). (c) Kaplan-Meier
3 survival curves of patients classified by high or low ATX expression between tumour and
4 paired non-tumour tissues (log-rank test, $p = 0.198$).

5 **Fig.4. Autotaxin promotes HCV infection.** For all experiments Huh-7 cells were cultured
6 under serum-free conditions for 8h prior to experimentation. (a) Huh-7 cells were treated
7 with HA130 (100nM) for 24h, LPA expression and cell viability (MTT) measured ($*p < 0.05$).
8 (b) Huh-7 were treated with HA130 for 1h prior to infecting with HCV J6/JFH or SA13/JFH for
9 24h ($*p < 0.05$; $***p < 0.001$). (c) ATX mRNA/protein expression and HCV infection of shATX
10 and Control silenced Huh-7 cells ($***p < 0.001$). (d) Control and ATX-silenced Huh-7 cells
11 were transfected with plasmids expressing ATX-wt or ATX-T210A and 48h later ATX secretion
12 assessed by western blot and cells infected with HCV for 24h ($*p < 0.05$; $**p < 0.01$). (e)
13 Control or ATX-silenced Huh-7 cells were treated with HA130 (100nM, 1h) or LPA (10uM,
14 15min) prior to infecting with HCV for 24h ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$). (f) Liver
15 specific differentiation markers (albumin, CYP3A4 and HNF4- α) and ATX mRNA levels were
16 measured in untreated and DMSO differentiated growth-arrested Huh-7 cells ($***p < 0.001$).
17 Differentiated cells were incubated with HA130 (100nM) for 1h prior to infecting with HCV
18 SA13/JFH. Infectivity was quantified by measuring the frequency of NS5A expressing cells
19 and expressed relative to untreated cells ($***p < 0.001$). All data sets are representative of at
20 least two independent experiments. Results in (a) show the mean value ± 1 standard error
21 of the mean (SEM), where the P values were obtained using two-way ANOVA accounting for
22 experimental variability on IBM SPSS Statistics Version 22.

23 **Fig.5. LPA regulates HCV infection.** Huh-7 cells were cultured under serum-free
24 conditions for 8h and (a) treated with LPA for 15mins prior to infecting with HCV J6/JFH or
25 SA13/JFH for 24h ($*p < 0.05$; $***p < 0.001$) or (b) incubated with Ki16425 (10uM) for
26 30mins prior to infecting with HCV J6/JFH or SA13/JFH ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).
27 Infectivity is expressed relative to the untreated samples and represents the mean of three
28 replicate infections. Infectivity data is representative of two independent experiments. (c)
29 LPAR mRNA expression levels in PHH (4 donors) and Huh-7 cells expressed relative to
30 GAPDH.

31 **Fig.6. ATX-LPA signalling regulates HCV RNA replication.** For all experiments Huh-7
32 cells were cultured under serum-free conditions for 8h prior to experimentation. (a) Huh-7
33 cells were incubated with HA130 (100nM) for 1h prior to infecting with HCVpp expressing
34 strain H77 or 1A38 envelope glycoproteins for 24h. Infectivity is expressed relative to
35 untreated cells and represents the mean of three replicate infections. (b) Huh-7 cells stably
36 expressing the Luc2A-JFH replicon were treated with HA130 (100nM) or transduced to
37 express shControl or shATX for 24h, cells lysed and luciferase activity measured. Data is
38 expressed relative to untreated and represents the mean of three replicate infections
39 ($***p < 0.001$). (c) Huh-7 cells were transfected with HCV J6/JFH or a polymerase replication
40 defective genome (GND⁻) that encodes gaussia luciferase (GLuc) and the cells grown under
41 serum-free conditions for 8h prior to treating with HA130 (100nM). Extracellular media was
42 collected at 0 and 24h post HA130 treatment and luciferase activity measured
43 ($***p < 0.001$). Viral replication is assessed by determining the ratio of J6/JFH/GND⁻
44 luciferase activity and represents the mean of three replicate infections.

45 **Fig.7. LPA stabilizes HIF-1 α via a PI3K dependent pathway.** For all experiments Huh-7
46 cells were cultured under serum-free conditions for 8h prior to experimentation. (a) Huh-7
47 cells expressing HRE-luciferase reporter were treated with LPA (10 μ M) in the presence or
48
49
50
51
52
53
54
55
56
57

1 absence of LPA receptor antagonist Ki16425 (10 μ M) for 24h. HIF-1 α expression was
2 measured by Western blotting (***p <0.001). **(b)** Huh-7 expressing HRE-luciferase reporter
3 were treated with NSC-134754 and cultured under normoxia or 1% oxygen for 24h, lysed
4 and HIF-transcriptional reporter activity measured (relative light units, RLU). Huh-7 cells
5 were treated with 10uM LPA (10 μ M) in the presence or absence of a sub-saturating dose of
6 NSC-134754 (25nM) for 15mins prior to infecting with HCV SA13/JFH for 24h and infectivity
7 assessed by enumerating NS5A expressing cells. **(c)** Huh-7 cells were treated with LPA
8 (10uM) and cell lysates (40g) probed for phospho-AKT (pAKT) or total AKT (AKT). **(d)** Huh-7
9 cells were treated with LPA (10 μ M) in the presence or absence of Ki16425 (10 μ M),
10 wortmannin (WM) (200nM), BYL-779 (2M) or TGX-221 (50nM) for 24h and cell lysates
11 (40 μ g) probed for HIF-1 α . **(e)** Huh-7 cells were treated with WM (200nM), BYL-779 (2 μ M) or
12 TGX-221 (50nM) for 15mins prior to infection with HCV J6/JFH in the presence or absence of
13 LPA (10 μ M). Infectivity is expressed relative to untreated cells and represents the mean of
14 three replicate infections (***p<0.001). HCV infection was measured by enumerating the
15 frequency of NS5A expressing cells 24h post inoculation (***p<0.001). **(f)** Schematic model
16 of ATX-LPA signaling axis in HCV replicative life cycle.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Materials and Methods

Cell lines, antibodies and reagents. Huh-7 (provided by Charles Rice, The Rockefeller University, NY, USA) and 293T (American Type Culture Collection) cells were propagated in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% non-essential amino acids (Invitrogen, CA). Huh-7 Luc2a-JFH cells (provided by Robert Thimme, Freiburg)[21] were propagated in the same media supplemented with G418. All cells were grown at 37°C in 5% CO₂. For hypoxic conditions cells were cultured at 37°C in a humidified sealed H35 Hypoxystation (Don Wiley Scientific, UK) set to 5% CO₂/95% N₂/1% O₂.

The primary antibodies were: anti-NS5A 9E10 (C. Rice, Rockefeller University, USA); anti-ATX 4FAB; anti-AKT and anti-pAKT (Cell Signaling); anti-HIF-1 α (BD Biosciences, Europe). Secondary labelled antibodies: Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, CA); Horseradish peroxidase conjugated sheep anti-mouse and donkey anti-rabbit (GE Healthcare, UK) and anti-rat secondary antibodies (Jackson laboratories). Agonists, inhibitors and antagonists were obtained from the following sources: HA130 (Echelon Biosciences), LPA (Oleoyl-L-alpha-lysophosphatidic acid sodium salt) and wortmannin (Sigma), Ki16425 (Cayman Chemical), BYL-719 (Active Biochem) and TGX-221 (Cayman Chemicals). Cell lysates were quantified for protein content using a standard Bradford assay and 40 μ g of protein analysed. For quantitation of secreted ATX, 50ul of extracellular serum-free conditioned media harvested from a defined cell number was analysed.

Solvents and chemicals for lipids analysis were purchased from the following suppliers: 13:0 LPA (1-tridecanoyl-sn-glycero-3-phosphate in methanol, Avanti Polar lipids-Stratech Scientific Limited), butanol Chromasolv Plus for HPLC and ammonium formate for mass spectrometry (Sigma), acetonitrile and water ultra-gradient grade (Romil), formic acid Optima LC/MS grade (Fisher Scientific).

uPA-SCID mice infection and immunohistochemical ATX staining. uPA/SCID-bg mice were transplanted with PHHs at 3 weeks of age by intrasplenic injection as described[14]. Engraftment was assessed by measuring human serum albumin and inoculated with HCV J6/JFH (Jc1) virus. Mice were sacrificed at 16 weeks, the liver recovered and frozen for RNA extraction. Liver samples were fixed in formalin for immunostaining purposes. Experiments were performed at Inserm Unit 1110 animal facility according to local laws and approved by the ethical committee of Strasbourg (number AL/02/19/08/12 and AL/01/18/08/12). Sections (3 μ m) of formalin fixed paraffin-embedded liver tissue were deparaffinised, rehydrated and after a low temperature retrieval technique and immunostained for ATX using a Dako Autostainer. Bound antibody was detected with rabbit anti-rat secondary for 15mins, ImmPRESS rabbit secondary for 30mins and visualised using ImmPACT DAB (Vector Labs, UK) and counterstained with Meyers haematoxylin.

RT-PCR quantification of ATX and HIF-target genes. Gene amplification was performed in a single tube RT-PCR in accordance with manufacturer's guidelines (CellsDirect kit, Invitrogen, CA) and fluorescence monitored in a 7900HT real time PCR machine (ABI, CA). The housekeeping gene GAPDH was included as an internal control for amplification efficiency and RNA quantification (primer-limited endogenous control, ABI).

HCC liver samples, clinical data and gene expression data. We used an Affymetrix U133A2.0 gene expression data set derived from 247 HCC patients as described[16] (GSE14520). Patient samples were obtained with informed consent from patients at the Liver Cancer Institute and Zhongshan Hospital (Fudan University, Shanghai, China). This cohort

1 contained paired tumour and adjacent non-tumour samples from 232 patients. We also
2 performed gene expression analysis of a German cohort from Heidelberg University
3 Hospital[15]. These tissues included tumour tissue of HCC patients with underlying alcoholic
4 liver disease (ALD, N=8) and HBV (N=8) or HCV (N=9) infection and normal liver samples of
5 patients without HCC or liver cirrhosis (NL, N=7).

6 **Ex vivo liver slices.** Liver tissue was obtained from patients undergoing resection or
7 transplantation surgery at the Queen Elizabeth Hospital, Birmingham. All liver samples were
8 collected with local National Health Service research ethics committee approval (Walsall LREC
9 04/Q2708/40) and written informed consent. Cores were cut from the tissue immediately
10 upon receipt in the laboratory. A Krumdieck Tissue Slicer (Alabama Research and
11 Development, USA) was used to section the liver cores. Briefly, the core was placed into the
12 slicer under aseptic conditions and circular slices of ~240µm thickness generated. Slices
13 were immediately transferred into Williams E media (Sigma, UK) supplemented with 1% L-
14 glutamine and 0.5µM insulin.

15
16
17
18 **Autotaxin promoter activity.** Forward (5'CCGGTACCTGTGCTGCGGAAGAAAAGATG3') and
19 reverse (5'GCCTCGAGGAAAGCCTTTAGCGTG3') primers were used to amplify the ATX
20 promoter region from HepG2 genomic DNA. PCR fragments were cloned into luciferase
21 reporter plasmid pGL4.28 (Promega, Madison, WI) digested with KpnI and XhoI
22 (pGL4.ATX.luc). Huh-7 cells were transfected with pGL4.ATX.luc or pHRE-Luc and 24h later
23 re-seeded in 96 well plates and incubated under 20% O₂ or 1% O₂ for 24h (Don Whitley
24 Scientific Limited). Cells were lysed and luminescence measured.

25
26
27
28 **Mass spectrometric LPA analysis.** Huh-7 cells were serum starved for 8h and
29 supernatants harvested, clarified and spiked with 1 ng of 13:0 LPA as an internal standard
30 prior to extraction with 500 µl of butanol. The combined butanol layers were dried under
31 reduced pressure and re-suspended in 100 µl of chloroform/methanol/water 2:5:1 (v/v/v). 5
32 µl of each sample was analyzed using a Shimadzu Prominence HPLC connected to a QTrap
33 equipped with an electrospray ionisation source (AB Sciex 6500). Separation of LPA species
34 from other interfering lipids such as LPS and LPC was achieved using a Cogent Diamond
35 Hydride column (1 x 150 mm, 4 µm, Microsolv) with the following conditions: 0.2 ml/min
36 flow rate, column temperature 40 oC, autosampler temperature 21 oC. Solvent A was 5 mM
37 ammonium formate aqueous solution pH 3.5 and solvent B was acetonitrile containing 0.1%
38 formic acid and 1% of a 200 mM aqueous solution of ammonium formate pH 3.5. Gradient
39 elution was as follows: isocratic 100% B for 4 minutes, linear decrease 100-75% B in 1.5
40 minutes, isocratic 75% B for 3.5 minutes, sharp step down to 25% B and isocratic 25% B for
41 5 minutes (washing step), followed by 10 minutes of re-equilibration with 100% B. The mass
42 spectrometer was operated in negative ion mode using multiple reaction monitoring to record
43 the following transitions: 367.2 → 153.0 for 13:0 LPA, 381.2 → 153.0 for 14:0 LPA, 409.2 →
44 153.0 for 16:0 LPA, 437.3 → 153.0 for 18:0 LPA, 435.3 → 153.0 for 18:1 LPA, 433.2 →
45 153.0 for 18:2 LPA, 457.2 → 153.0 for 20:4 LPA, 481.2 → 153.0 for 22:6 LPA. The following
46 optimized MS conditions were used for the analysis: Curtain Gas, 20 psi; Collision Activated
47 Dissociation, Medium; Ion Spray Voltage, -4500 V; Ion Source Temperature, 400 oC; Ion
48 Source Gas 1, 40 psi; Ion Source Gas 2, 30 psi; Declustering Potential, -110 V; Collision
49 Energy, -30 eV; Dwell Time, 50 ms.

50
51
52
53
54
55
56
57 **HCV genesis and quantification of infection.** HCV was generated using the Megascript T7
58 kit (Ambion, Austin, TX), RNA was transcribed in vitro from full-length genomes and
59 electroporated into Huh-7 cells. After 48h cells were serum starved for 8h prior to collecting
60 the serum-free media at 72h post infection and storing at -80°C. Pseudoviruses expressing
61
62
63
64
65

1 luciferase reporters were generated following transfection of 293T cells with a 1:1 ratio of
2 plasmids encoding HIV provirus expressing luciferase and HCV strain 1A38 or H77 E1E2
3 envelope gps (HCVpp-1A38, HCVpp-H77) or empty vector (Env-pp). At 24h post transfection
4 cells were serum starved and the pseudoparticles harvested 48h post transfection in serum
5 free media.

6 Target cells were seeded at 1.5×10^4 cells/cm² and serum starved for 8h prior to infection
7 with HCV or HCVpp in serum-free media for 24h. HCV infection was assessed following
8 methanol fixation and staining for NS5A with 9E10 antibody; bound antibody was detected
9 with an Alexa 488-conjugated anti-mouse IgG and quantified by enumerating NS5A⁺ cells.
10 Pseudoparticle infection was quantified by measuring cellular luciferase activity in a
11 luminometer (Berthold Centro LB 960). Relative infectivity was calculated as a percentage of
12 untreated cells and presented \pm standard error of the mean (SEM), where the mean infection
13 value of replicate untreated cells wells was defined as 100%.
14
15

16
17 **Assessing the role of ATX in the HCV life cycle.** Target cells were seeded at $1.5 \times$
18 10^4 /cm² and serum starved for 8h prior to infection. Cells were incubated with HA130
19 (60min), LPA (in the presence of 0.1mg/ml fatty acid free BSA) (15min), Ki16425 or LPA
20 plus Ki16425 (15min) diluted in serum-free media prior to infecting with HCV or HCVpp for
21 24h. Huh-7 Luc2a-JFH expressing cells or shATX/shControl transduced cells were seeded at
22 1.5×10^4 /cm² and serum starved for 8h prior to assay. Cells were untreated or incubated
23 with HA130 for 24h prior to lysis and measuring luciferase. Huh-7 cells were transfected with
24 JC1GLuc for 24h, serum starved for 8h and washed extensively before treating with HA130
25 for 24h. Extracellular media was harvested, heat inactivated and luciferase activity
26 measured.
27
28
29
30

31 **Autotaxin silencing, rescue, and HIF-1 α silencing.** 293T cells were transfected with
32 plasmids pIKO.1 shATX ([TRCN0000048993](#)) or pIKO.1 control (Open Biosystems) plus p8.2
33 gag pol and VSV-G and 72h later lentiviral particles harvested and used to transduce Huh-7.
34 Cells were cultured in the presence of puromycin and ATX knockdown determined by
35 Western blotting. shControl and shATX stable expressing Huh-7 cells were transfected with
36 His-tagged wild type ATX (ATX-wt) or T210A ATX mutant (ATX-T210A) (Addgene plasmid
37 17839 and 17840 respectively) for 24h prior to seeding cells for infection studies. Huh-7 cells
38 were transfected using Dharmafect according to manufactures instructions with siHIF-1 α
39 (siHIF) or shControl (Thermoscientific, UK) and incubated for 48h prior assessing HIF-1 α by
40 Western blot or infecting with HCV.
41
42
43

44 **Statistical analysis.** Results are shown as the mean value \pm 1 standard deviation (SD)
45 except where stated otherwise, all data were tested for fit a Gaussian assumptions and
46 analyses performed using either Student's t-test (pairwise comparisons) or Kruskal-Wallis
47 One-Way ANOVA with Dunn's test (for multiple comparisons), except where stated
48 otherwise, in Graph Pad Prism 6 (GraphPad, USA), with a P value of <0.05 considered
49 statistically significant. Expression differences between HCC and non-tumorous liver samples
50 were assessed by Wilcoxon signed-rank test for paired samples. Kaplan-Maier curves and
51 log-rank test were performed with Graph Pad Prism 6. Differential expression in aetiology
52 groups of the German cohort were analysed by nonparametric Mann-Whitney U tests. The
53 hypoxia score was calculated as previously reported[17].
54
55
56
57
58
59
60
61
62
63
64
65

References:

- 1 [1] Pawlotsky JM. New hepatitis C virus (HCV) drugs and the hope for a cure:
2 concepts in anti-HCV drug development. *Seminars in liver disease* 2014;34:22-29.
- 3 [2] van der Meer AJ, Veldt BJ, Feld JJ, Wedemeyer H, Dufour JF, Lammert F, et
4 al. Association between sustained virological response and all-cause mortality
5 among patients with chronic hepatitis C and advanced hepatic fibrosis. *JAMA*
6 2012;308:2584-2593.
- 7 [3] **Reig M, Marino Z**, Perello C, Inarrairaegui M, Ribeiro A, Lens S, et al.
8 Unexpected early tumor recurrence in patients with hepatitis C virus -related
9 hepatocellular carcinoma undergoing interferon-free therapy: a note of caution.
10 *Journal of hepatology* 2016.
- 11 [4] Stracke ML, Krutzsch HC, Unsworth EJ, Arestad A, Cioce V, Schiffmann E, et
12 al. Identification, purification, and partial sequence analysis of autotaxin, a novel
13 motility-stimulating protein. *The Journal of biological chemistry* 1992;267:2524-
14 2529.
- 15 [5] **Barbayianni E, Kaffe E**, Aidinis V, Kokotos G. Autotaxin, a secreted
16 lysophospholipase D, as a promising therapeutic target in chronic inflammation and
17 cancer. *Prog Lipid Res* 2015;58:76-96.
- 18 [6] Schlatzer DM, Sugalski JM, Chen Y, Barnholtz-Sloan J, Davitkov P, Hazlett
19 FE, et al. Plasma proteome analysis reveals overlapping, yet distinct mechanisms of
20 immune activation in chronic HCV and HIV infections. *Journal of acquired immune*
21 *deficiency syndromes* 2013;63:563-571.
- 22 [7] Wu JM, Xu Y, Skill NJ, Sheng H, Zhao Z, Yu M, et al. Autotaxin expression
23 and its connection with the TNF-alpha-NF-kappaB axis in human hepatocellular
24 carcinoma. *Molecular cancer* 2010;9:71.
- 25 [8] Cooper AB, Wu J, Lu D, Maluccio MA. Is autotaxin (ENPP2) the link between
26 hepatitis C and hepatocellular cancer? *Journal of gastrointestinal surgery : official*
27 *journal of the Society for Surgery of the Alimentary Tract* 2007;11:1628-1634;
28 discussion 1634-1625.
- 29 [9] Kondo M, Ishizawa T, Enooku K, Tokuhara Y, Ohkawa R, Uranbileg B, et al.
30 Increased serum autotaxin levels in hepatocellular carcinoma patients were caused
31 by background liver fibrosis but not by carcinoma. *Clinica chimica acta; international*
32 *journal of clinical chemistry* 2014;433:128-134.
- 33 [10] Pleli T, Martin D, Kronenberger B, Brunner F, Koberle V, Grammatikos G, et
34 al. Serum autotaxin is a parameter for the severity of liver cirrhosis and overall
35 survival in patients with liver cirrhosis--a prospective cohort study. *PloS one*
36 2014;9:e103532.
- 37 [11] Mazzocca A, Dituri F, Lupo L, Quaranta M, Antonaci S, Giannelli G. Tumor-
38 secreted lysophosphatidic acid accelerates hepatocellular carcinoma progression by
39 promoting differentiation of peritumoral fibroblasts in myofibroblasts. *Hepatology*
40 2011;54:920-930.
- 41 [12] Nasimuzzaman M, Waris G, Mikolon D, Stupack DG, Siddiqui A. Hepatitis C
42 virus stabilizes hypoxia-inducible factor 1alpha and stimulates the synthesis of
43 vascular endothelial growth factor. *Journal of virology* 2007;81:10249-10257.
- 44 [13] Wilson GK, Brimacombe CL, Rowe IA, Reynolds GM, Fletcher NF, Stamataki
45 Z, et al. A dual role for hypoxia inducible factor-1alpha in the hepatitis C virus
46 lifecycle and hepatoma migration. *Journal of hepatology* 2012;56:803-809.
- 47 [14] Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al.
48 Hepatitis C virus replication in mice with chimeric human livers. *Nature medicine*
49 2001;7:927-933.
- 50 [15] Neumann O, Kesselmeier M, Geffers R, Pellegrino R, Radlwimmer B,
51 Hoffmann K, et al. Methylome analysis and integrative profiling of human HCCs
52 identify novel protumorigenic factors. *Hepatology* 2012;56:1817-1827.
- 53 [16] Roessler S, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, et al. A unique
54 metastasis gene signature enables prediction of tumor relapse in early-stage
55 hepatocellular carcinoma patients. *Cancer research* 2010;70:10202-10212.
- 56
- 57
- 58
- 59
- 60
- 61
- 62
- 63
- 64
- 65

- 1 [17] van Malenstein H, Gevaert O, Libbrecht L, Daemen A, Allemeersch J, Nevens
2 F, et al. A seven-gene set associated with chronic hypoxia of prognostic importance
3 in hepatocellular carcinoma. *Clinical cancer research : an official journal of the*
4 *American Association for Cancer Research* 2010;16:4278-4288.
- 5 [18] Albers HM, Dong A, van Meeteren LA, Egan DA, Sunkara M, van Tilburg EW,
6 et al. Boronic acid-based inhibitor of autotaxin reveals rapid turnover of LPA in the
7 circulation. *Proc Natl Acad Sci U S A* 2010;107:7257-7262.
- 8 [19] Sainz B, Jr., Chisari FV. Production of infectious hepatitis C virus by well-
9 differentiated, growth-arrested human hepatoma-derived cells. *Journal of virology*
10 2006;80:10253-10257.
- 11 [20] Ohta H, Sato K, Murata N, Damirin A, Malchinkhuu E, Kon J, et al. Ki16425, a
12 subtype-selective antagonist for EDG-family lysophosphatidic acid receptors.
13 *Molecular pharmacology* 2003;64:994-1005.
- 14 [21] Bailey JR, Wasilewski LN, Snider AE, El-Diwany R, Osburn WO, Keck Z, et al.
15 Naturally selected hepatitis C virus polymorphisms confer broad neutralizing
16 antibody resistance. *J Clin Invest* 2015;125:437-447.
- 17 [22] Lee SJ, No YR, Dang DT, Dang LH, Yang VW, Shim H, et al. Regulation of
18 hypoxia-inducible factor 1alpha (HIF-1alpha) by lysophosphatidic acid is dependent
19 on interplay between p53 and Kruppel-like factor 5. *The Journal of biological*
20 *chemistry* 2013;288:25244-25253.
- 21 [23] Baker LC, Boulton JK, Walker-Samuel S, Chung YL, Jamin Y, Ashcroft M, et al.
22 The HIF-pathway inhibitor NSC-134754 induces metabolic changes and anti-tumour
23 activity while maintaining vascular function. *Br J Cancer* 2012;106:1638-1647.
- 24 [24] Lee J, Park SY, Lee EK, Park CG, Chung HC, Rha SY, et al. Activation of
25 hypoxia-inducible factor-1alpha is necessary for lysophosphatidic acid-induced
26 vascular endothelial growth factor expression. *Clinical cancer research : an official*
27 *journal of the American Association for Cancer Research* 2006;12:6351-6358.
- 28 [25] Mannova P, Beretta L. Activation of the N-Ras-PI3K-Akt-mTOR pathway by
29 hepatitis C virus: control of cell survival and viral replication. *Journal of virology*
30 2005;79:8742-8749.
- 31 [26] Chen MH, Lee MY, Chuang JJ, Li YZ, Ning ST, Chen JC, et al. Curcumin
32 inhibits HCV replication by induction of heme oxygenase-1 and suppression of AKT.
33 *International journal of molecular medicine* 2012;30:1021-1028.
- 34 [27] Pisonero-Vaquero S, Garcia-Mediavilla MV, Jorquera F, Majano PL, Benet M,
35 Jover R, et al. Modulation of PI3K-LXRalpha-dependent lipogenesis mediated by
36 oxidative/nitrosative stress contributes to inhibition of HCV replication by quercetin.
37 *Laboratory investigation; a journal of technical methods and pathology*
38 2014;94:262-274.
- 39 [28] Vassilaki N, Kalliampakou KI, Kotta-Loizou I, Befani C, Liakos P, Simos G, et
40 al. Low oxygen tension enhances hepatitis C virus replication. *Journal of virology*
41 2013;87:2935-2948.
- 42 [29] Binder BY, Genetos DC, Leach JK. Lysophosphatidic acid protects human
43 mesenchymal stromal cells from differentiation-dependent vulnerability to
44 apoptosis. *Tissue engineering Part A* 2014;20:1156-1164.
- 45 [30] Kostic I, Fidalgo-Carvalho I, Aday S, Vazao H, Carvalho T, Graos M, et al.
46 Lysophosphatidic acid enhances survival of human CD34(+) cells in ischemic
47 conditions. *Scientific reports* 2015;5:16406.
- 48 [31] Liu LP, Hu BG, Ye C, Ho RL, Chen GG, Lai PB. HBx mutants differentially
49 affect the activation of hypoxia-inducible factor-1alpha in hepatocellular carcinoma.
50 *Br J Cancer* 2014;110:1066-1073.
- 51 [32] Szabo G. Gut-liver axis in alcoholic liver disease. *Gastroenterology*
52 2015;148:30-36.
- 53 [33] Jansen S, Andries M, Vekemans K, Vanbilloen H, Verbruggen A, Bollen M.
54 Rapid clearance of the circulating metastatic factor autotaxin by the scavenger
55 receptors of liver sinusoidal endothelial cells. *Cancer letters* 2009;284:216-221.
- 56 [34] Muro H, Shirasawa H, Kosugi I, Nakamura S. Defect of Fc receptors and
57 phenotypical changes in sinusoidal endothelial cells in human liver cirrhosis. *The*
58 *American journal of pathology* 1993;143:105-120.
- 59
60
61
62
63
64
65

- 1 [35] Baumforth KRN, Flavell JR, Reynolds GM, Davies G, Pettit TR, Wei WB, et al. Induction of autotaxin by the Epstein-Barr virus promotes the growth and survival
2 of Hodgkin lymphoma cells. *Blood* 2005;106:2138-2146.
- 3 [36] Kondo S, Seo SY, Yoshizaki T, Wakisaka N, Furukawa M, Joab I, et al. EBV
4 latent membrane protein 1 up-regulates hypoxia-inducible factor 1alpha through
5 Siah1-mediated down-regulation of prolyl hydroxylases 1 and 3 in nasopharyngeal
6 epithelial cells. *Cancer research* 2006;66:9870-9877.
- 7 [37] Tigyi G. Aiming drug discovery at lysophosphatidic acid targets. *Br J*
8 *Pharmacol* 2010;161:241-270.
- 9 [38] Budd DC, Qian Y. Development of lysophosphatidic acid pathway modulators
10 as therapies for fibrosis. *Future medicinal chemistry* 2013;5:1935-1952.
- 11 [39] Mazzocca A, Dituri F, De Santis F, Filannino A, Lopane C, Betz RC, et al.
12 Lysophosphatidic acid receptor LPAR6 supports the tumorigenicity of hepatocellular
13 carcinoma. *Cancer research* 2015;75:532-543.
- 14 [40] Brindley DN, Lin FT, Tigyi GJ. Role of the autotaxin-lysophosphatidate axis in
15 cancer resistance to chemotherapy and radiotherapy. *Biochimica et biophysica acta*
16 2013;1831:74-85.
- 17 [41] Barbayianni E, Magrioti V, Moutevelis-Minakakis P, Kokotos G. Autotaxin
18 inhibitors: a patent review. *Expert opinion on therapeutic patents* 2013;23:1123-
19 1132.
- 20 [42] van Meeteren LA, Ruurs P, Stortelers C, Bouwman P, van Rooijen MA,
21 Pradere JP, et al. Autotaxin, a secreted lysophospholipase D, is essential for blood
22 vessel formation during development. *Molecular and cellular biology* 2006;26:5015-
23 5022.
- 24 [43] Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, et al. Autotaxin
25 stabilizes blood vessels and is required for embryonic vasculature by producing
26 lysophosphatidic acid. *The Journal of biological chemistry* 2006;281:25822-25830.
- 27 [44] Fotopoulou S, Oikonomou N, Grigorieva E, Nikitopoulou I, Paparountas T,
28 Thanassopoulou A, et al. ATX expression and LPA signalling are vital for the
29 development of the nervous system. *Developmental biology* 2010;339:451-464.
- 30 [45] Katsifa A, Kaffe E, Nikolaidou-Katsaridou N, Economides AN, Newbigging S,
31 McKerlie C, et al. The Bulk of Autotaxin Activity Is Dispensable for Adult Mouse Life.
32 *PLoS one* 2015;10:e0143083.
- 33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

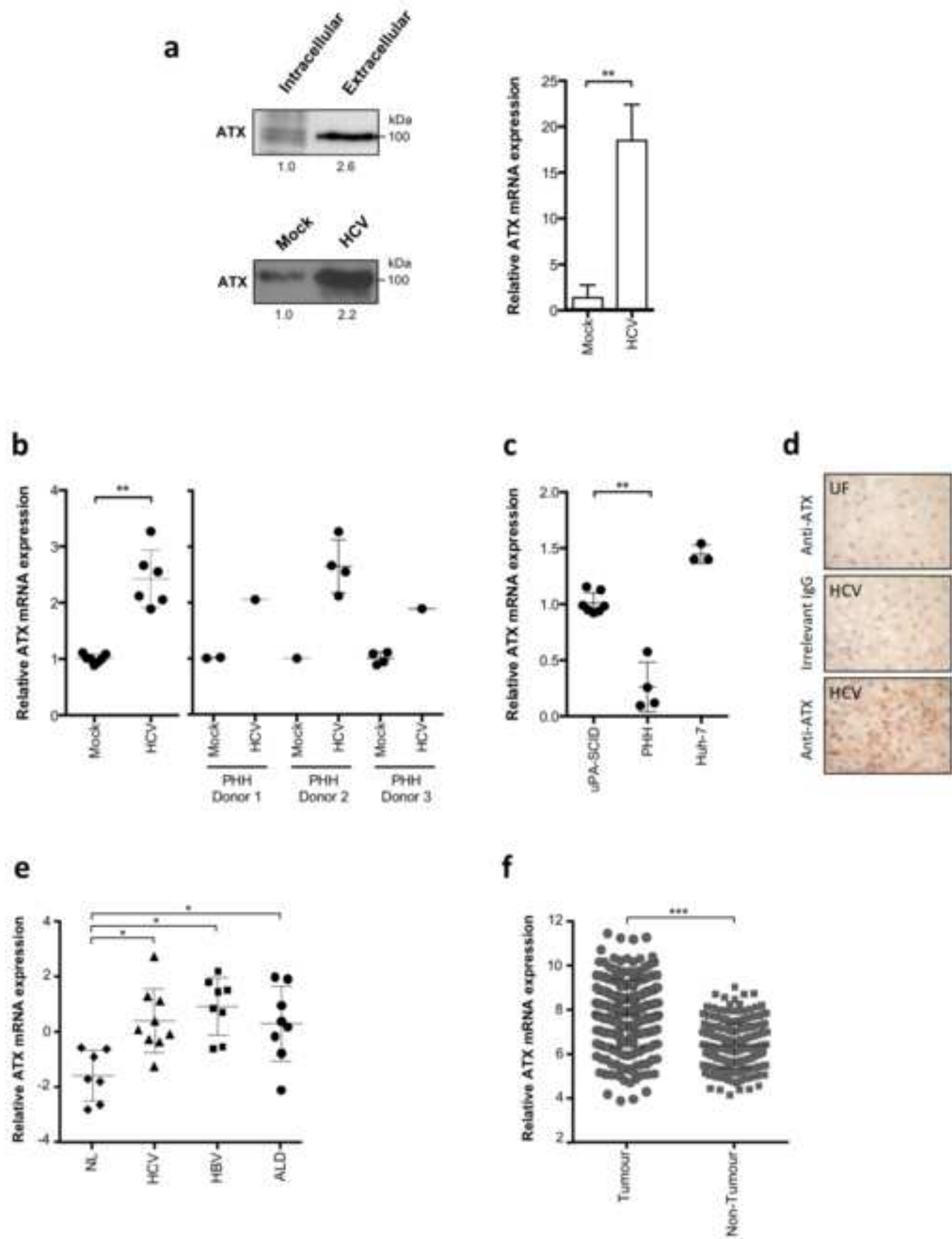
Figure 1[Click here to download high resolution image](#)

Figure 2
[Click here to download high resolution image](#)

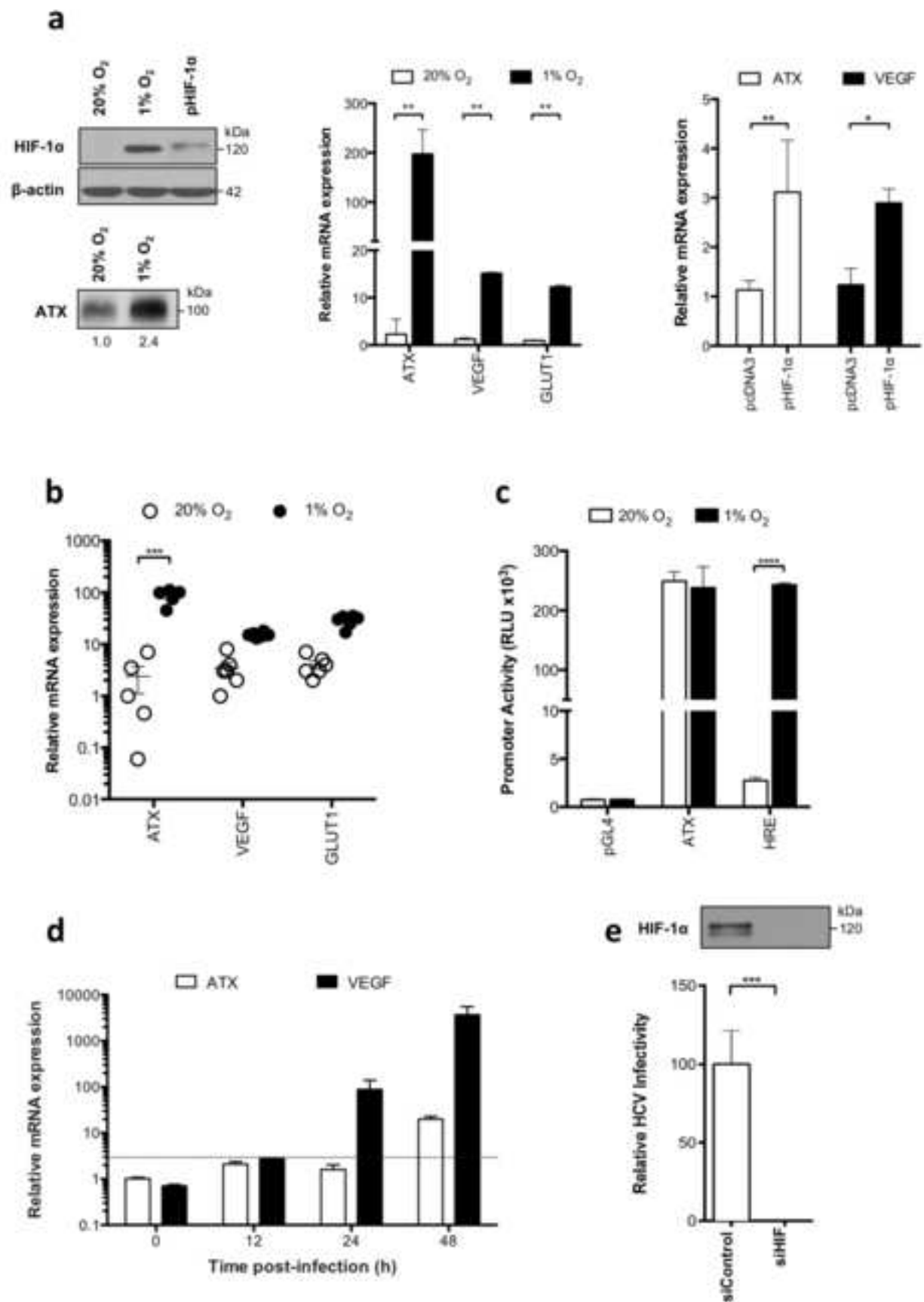


Figure 3
[Click here to download high resolution image](#)

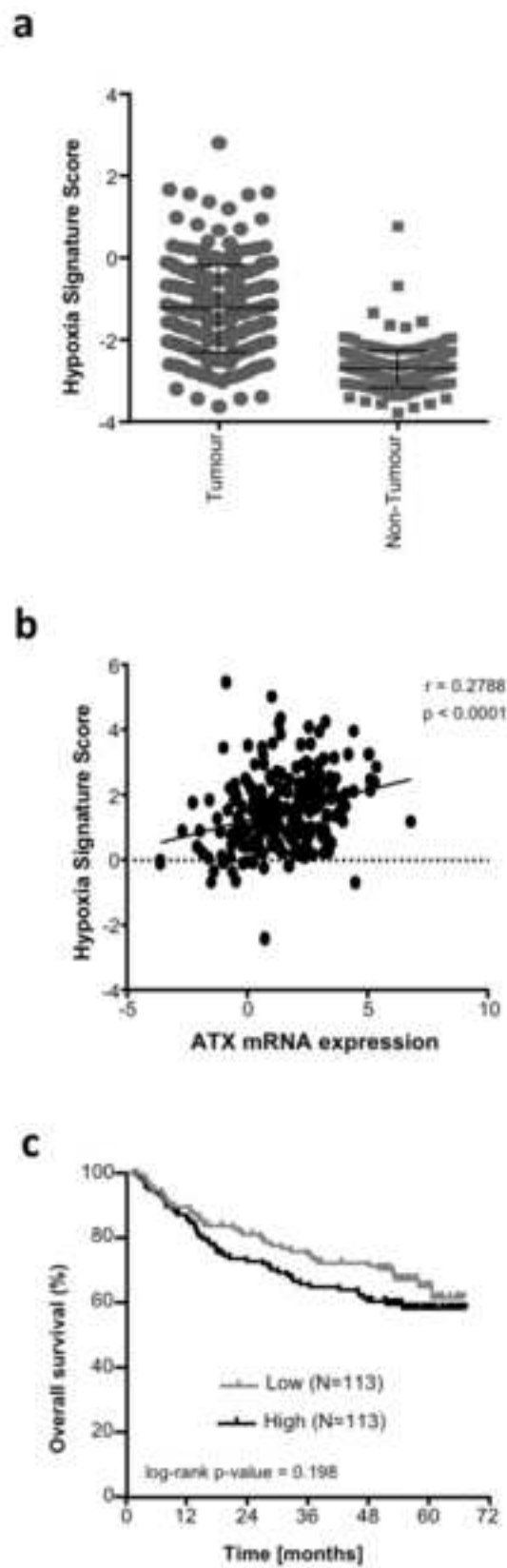


Figure 4
[Click here to download high resolution image](#)

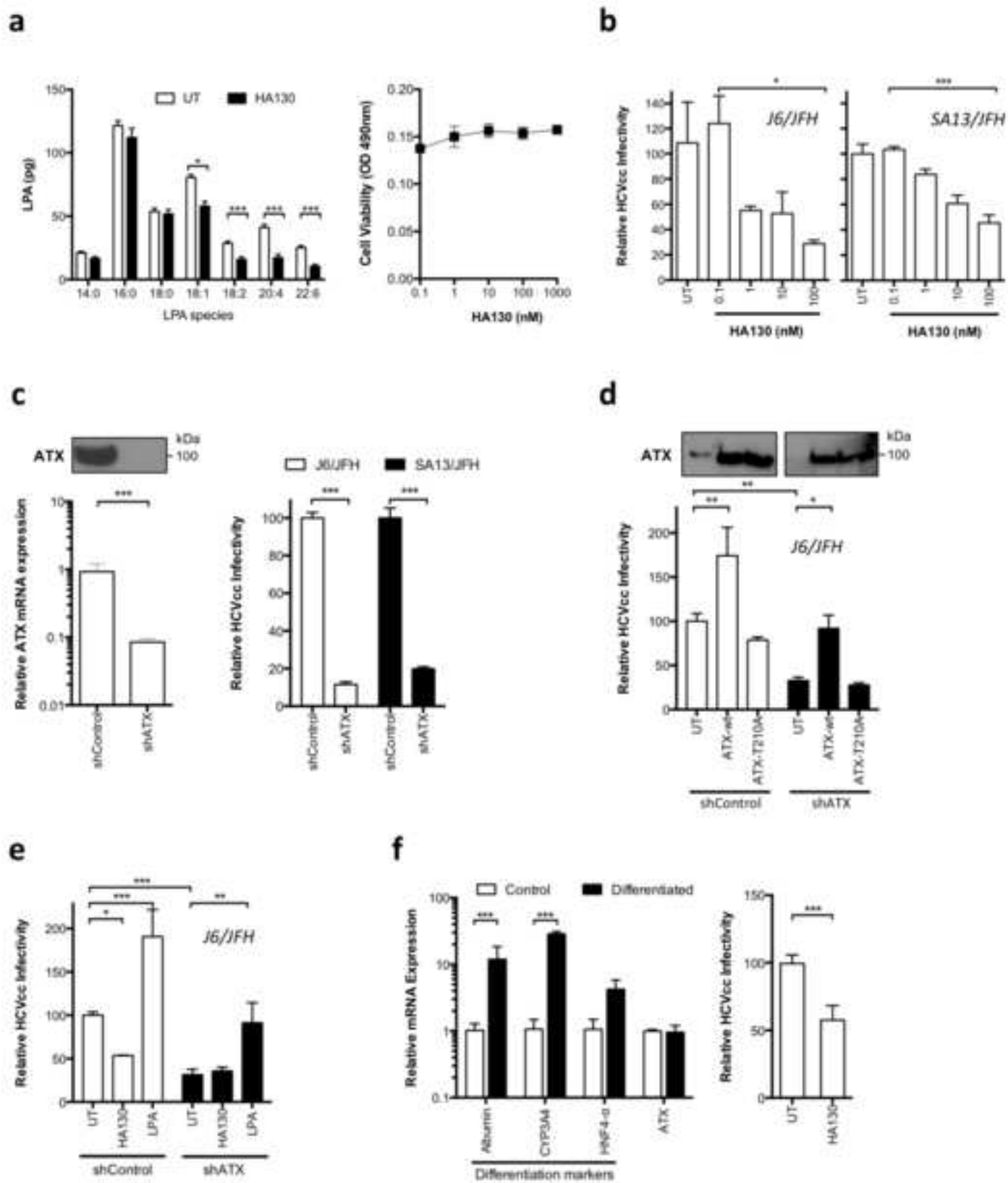


Figure 5

[Click here to download high resolution image](#)

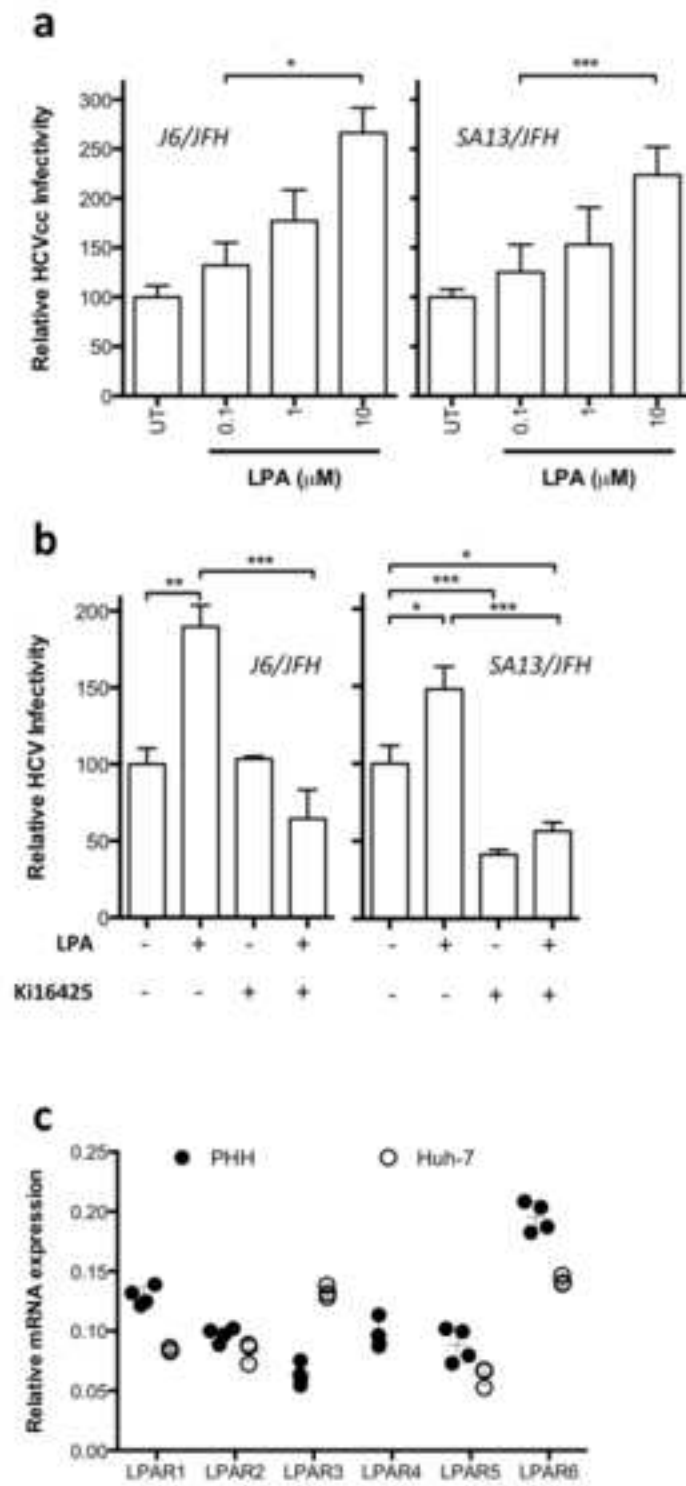


Figure 6
[Click here to download high resolution image](#)

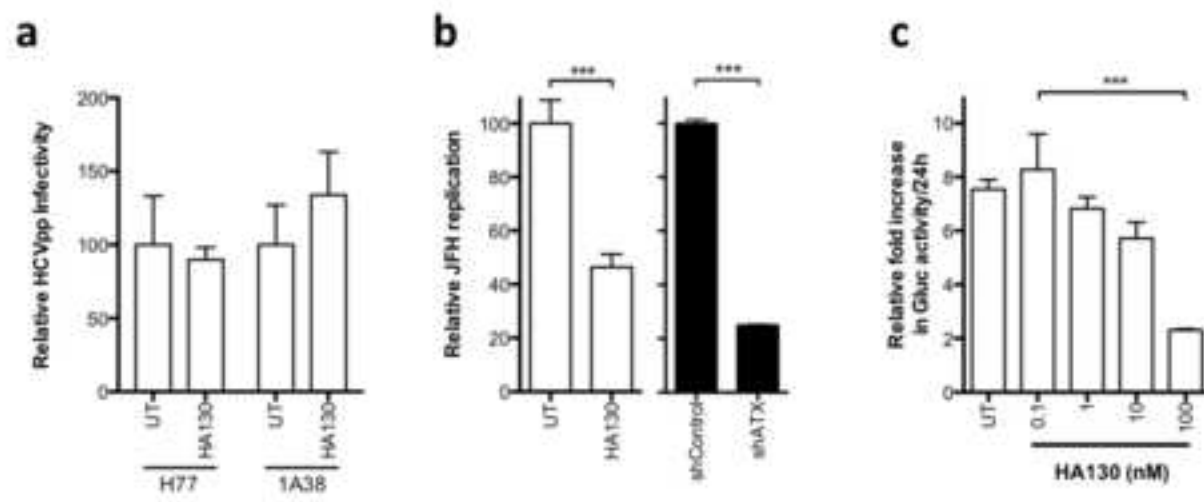
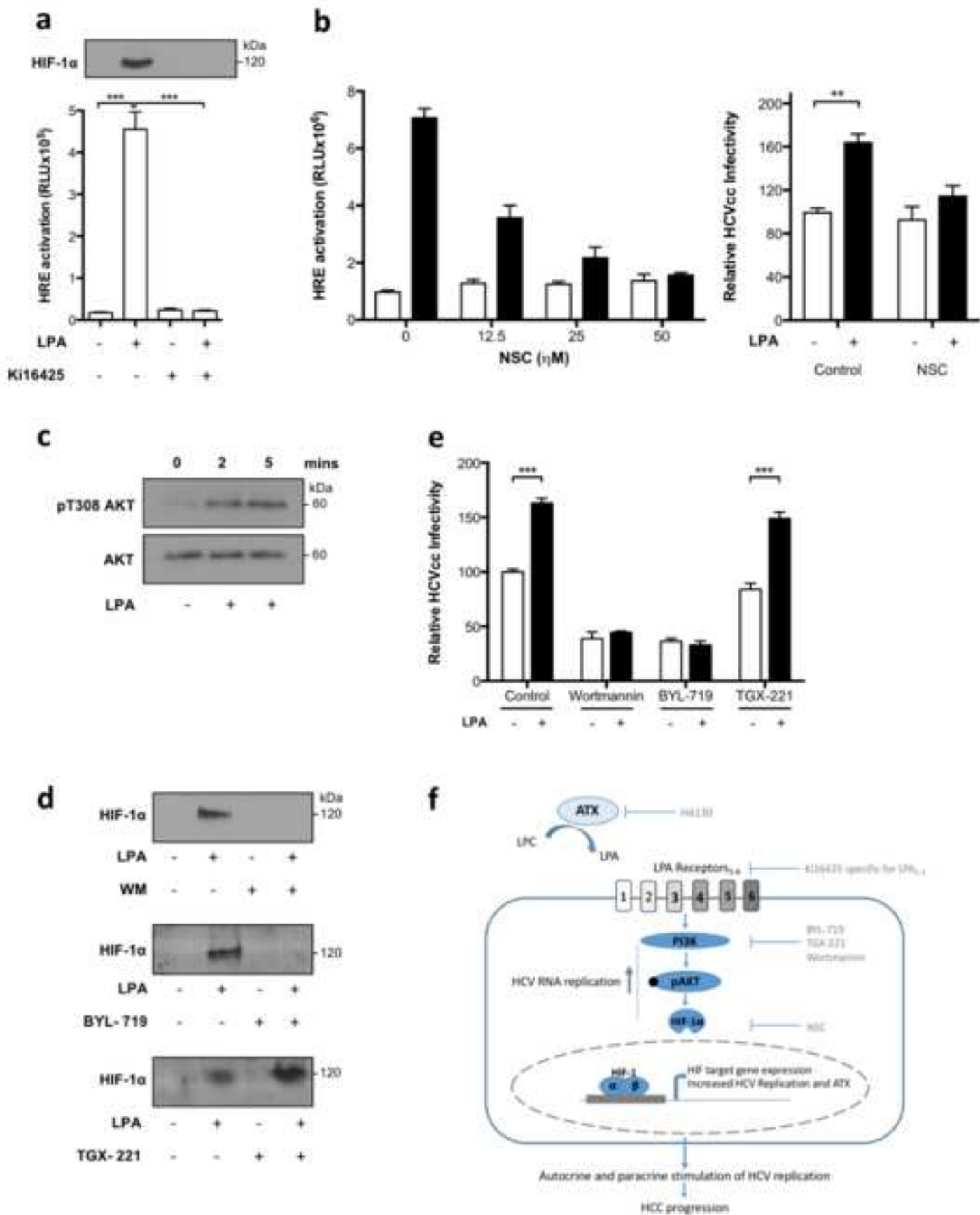
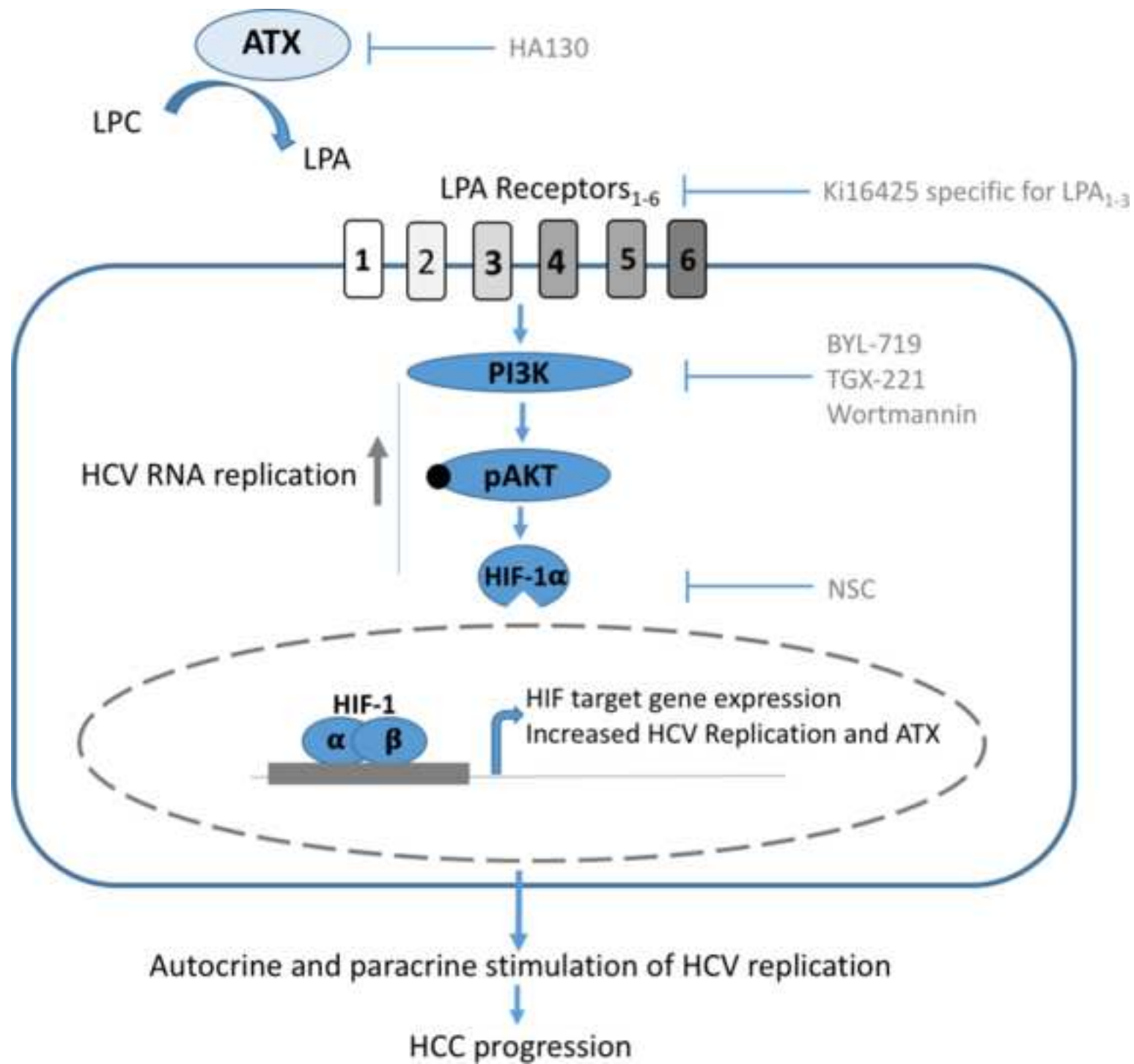


Figure 7
[Click here to download high resolution image](#)





Supplementary material

[Click here to download Supplementary material: Supplementary Figure.docx](#)

*ICMJE disclosure form

[Click here to download ICMJE disclosure form: JHEPAT-D-16-01414R1-ICMJE.pdf](#)