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Hepatitis C virus (HCV) protein expression enhances hepatic fibrosis in HCV transgenic mice exposed to a fibrogenic agent.

Philippe Chouteau, Nicole Defer, Alexandre Florimond, Julien Caldéraro, Martin Higgs, Aurore Gaudin, Emilie Mérour, Daniel Dhumeaux, Hervé Lerat, Jean-Michel Pawlotsky

► To cite this version:

Philippe Chouteau, Nicole Defer, Alexandre Florimond, Julien Caldéraro, Martin Higgs, et al.. Hepatitis C virus (HCV) protein expression enhances hepatic fibrosis in HCV transgenic mice exposed to a fibrogenic agent.. *Journal of Hepatology*, 2012, 57 (3), pp.499-507. 10.1016/j.jhep.2012.04.019 . inserm-00734273

HAL Id: inserm-00734273

<https://inserm.hal.science/inserm-00734273>

Submitted on 21 Sep 2012

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4 **Hepatitis C Virus (HCV) Protein Expression Enhances Hepatic Fibrosis**
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7 **in HCV Transgenic Mice Exposed to a Fibrogenic Agent**
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56
57 **Electronic word count:** 5946
58

59 **Abstract electronic word count:** 244
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4 | **Number of figures:** [6](#)

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6 | **Number of tables:** 1

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11 **List of abbreviations:** HCV, hepatitis C virus; ORF, open reading frame; ECM, extra-
12 cellular matrix; CCl₄, carbon tetrachloride; wt, wild-type; qPCR, quantitative PCR;
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14 hepatic stellate cells; HPCs, hepatic progenitor cells; PBS, phosphate buffer saline;
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16 CYP2E1, cytochrome P450 2E1; ROS, reactive oxygen species; BrdU,
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18 bromodeoxyuridine; ALT, alanine aminotransferase; AST, aspartate aminotransferase;
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28 CCL5, chemokine (C-C motif) ligand 5.

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31 **Conflict of interest:** The authors declare that they have nothing to disclose regarding
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35
36 **Financial Support:** This study was supported by the National Agency for Research on
37
38 AIDS and Viral Hepatitis (ANRS; R0711JJ and R09017JJ). A. F. is the recipient of a
39
40 postdoctoral fellowship from the French Ministry of Research; M. H. was the recipient of
41
42 a postdoctoral fellowship from the ANRS.
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ABSTRACT

Background & Aims: During chronic HCV infection, activation of fibrogenesis appears to be principally related to local inflammation. However, the direct role of hepatic HCV protein expression in fibrogenesis remains unknown.

Methods: We used transgenic mice expressing the full-length HCV open reading frame exposed to a 'second hit' of the fibrogenic agent carbon tetrachloride (CCl₄). Both acute and chronic liver injuries were induced in these mice by CCl₄ injections. Liver injury, expression of matrix re-modeling genes, reactive oxygen species (ROS), inflammation, hepatocyte proliferation, ductular reaction and hepatic progenitor cells (HPC) expansion were examined.

Results: After CCl₄ treatment, HCV transgenic mice exhibited enhanced liver fibrosis, significant changes in matrix re-modeling genes and increased ROS production compared to wt littermates despite no differences in the degree of local inflammation. This increase was accompanied by a decrease in hepatocyte proliferation, which appeared to be due to delayed hepatocyte entry into the S-phase. A prominent ductular reaction and hepatic progenitor cell compartment expansion were observed in transgenic animals. These observations closely mirror those previously made in HCV-infected individuals.

Conclusions: Together, these results demonstrate that expression of the HCV proteins in hepatocytes contributes to the development of hepatic fibrosis in the presence of other fibrogenic agents. In the presence of CCl₄, HCV transgenic mice display an intra-hepatic re-organization of several key cellular actors in the fibrogenic process.

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6 **Keywords:** hepatitis C virus; fibrosis; hepatocyte proliferation; reactive oxygen species;
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INTRODUCTION

Hepatic fibrosis, a result of active fibrogenesis, is a frequent feature of chronic hepatitis C virus (HCV) infection. Fibrogenesis is classically thought to be triggered principally by the local immune response. The role of HCV infection in fibrogenesis, in particular HCV protein expression in the liver, is unknown. Mice of the FL-N/35 lineage express the entire open reading frame (ORF) of a genotype 1b isolate of HCV in a liver-specific fashion [1]. In this model, the HCV proteins are expressed within hepatocytes at levels close to those observed in human infection, without infectious virus production or an antiviral immune response. These mice develop lesions similar to those observed during natural infection, including hepatic steatosis and hepatocellular carcinoma [2-6]. Preliminary observations suggested that FL-N/35 mice may be of interest in the study of liver fibrosis [1]. However, this model lacks the concomitant fibrogenesis triggers (such as liver inflammation) observed in HCV-infected individuals. Therefore, the effect of HCV protein expression on hepatic fibrosis in this model must be studied in the context of a 'second hit' of chronic liver injury.

The conventional paradigm of fibrogenesis in the liver is largely based on activation of fibrogenic processes associated with transformation of hepatic stellate cells (HSCs) into myofibroblast-like cells [7-10]. However, it remains paradoxical that, whereas HCV infection is present largely within hepatic lobules [11], fibrosis predominates in portal areas in chronically infected patients. Currently, there are no explanations for this observation, and the surrounding mechanisms remain

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4 uninvestigated. One possible explanation is that viral particles produced within
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6 intralobular hepatocytes might directly activate periportal HSCs. Another non-exclusive
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8 explanation is that HCV infection may be accompanied by ductular proliferation [12].
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11 In the normal liver, the replacement of necrotic and apoptotic hepatocytes occurs
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13 through replication of adjacent hepatocytes within the lobules. However, this primary
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15 pathway is impaired by a variety of insults, including viral infections [13, 14]. These
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17 situations have been shown to lead to proliferation of hepatic progenitor cells (HPCs)
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19 [15-17]. Such cells reside primarily in the periportal region and become the source of
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21 regenerating hepatocytes, as well as cholangiocytes and draining ductules [15, 18]. A
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23 by-product of the activation of this secondary proliferative pathway is a ductular reaction
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28 [15], which has been linked to fibrogenesis [19-28].
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31 A strong correlation has been reported between HCV-related portal fibrosis and a
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33 periportal ductular reaction, with a relationship between the number of proliferating
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35 HPCs and the number of hepatocytes in replication arrest [29]. These observations
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37 support the hypothesis that portal fibrosis during chronic HCV infection may result from
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39 a periportal ductular reaction, possibly as a consequence of altered hepatocyte
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41 proliferation, possibly as a consequence of altered hepatocyte
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43 proliferation.
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46 We illustrate below, using the FL-N/35 HCV transgenic mouse model in
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48 conjunction with an exogenous chemical fibrogenic agent, that the expression of HCV
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50 proteins *in vivo* plays a contributory role in the fibrogenic process, and is accompanied
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52 by a ductular reaction and a replicative arrest of intra-lobular hepatocytes concomitant
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54 with enhanced reactive oxygen species (ROS) production, in the absence of enhanced
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56 inflammation.
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MATERIALS AND METHODS

Chemicals and antibodies

Carbon tetrachloride (CCl₄) and olive oil were purchased from Sigma-Aldrich (St. Louis, MO). Primary antibodies are described in more details in Supplementary Materials and Methods.

Animals

Wild-type mice (wt) and those transgenic for the entire HCV ORF (FL-N/35 lineage) were from the same genetic background (C57/Bl6) [1]. They were used in conformation with the European Community Council directive. Animals were bred and housed as previously described [2]. Studies were performed on age-matched 7- to 10-month old males.

Chronic CCl₄ treatment

Animals were injected intraperitoneally twice a week for 4 weeks with CCl₄ (0.5 µg/g of body weight) diluted in 5% olive oil, obtained the day of injection. Control mice were injected with an equal volume of olive oil. Twenty-four hours after the final injection, animals were sacrificed by CO₂ inhalation, and body and liver weights were measured. Sera (100 µL/mouse) and livers were immediately processed for enzymatic

assays and histological determination, or kept at -80 °C until processing.

Experimental acute liver injury and measurement of hepatocyte DNA

synthesis

Mice were treated with a single intraperitoneal injection of CCl₄ (795 mg/kg body weight diluted in 5% olive oil). They were sacrificed at different time points after the injection. Bromodeoxyuridine (BrdU) was injected intraperitoneally (150 mg/kg of body weight) two hours prior to sacrifice. Hepatocyte DNA synthesis was measured by means of immunodetection of BrdU incorporation as described in Supplementary Materials and Methods.

Western blotting

Frozen liver tissues were processed as previously described [6]. The detailed procedure is described in Supplementary Materials and Methods.

Serum alanine aminotransferase and aspartate aminotransferase level measurements

Levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum of mice were measured on a Cobas[®]6000 analyzer (Roche, Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

Liver histology and immunohistochemistry

Fresh liver tissues were fixed in 10% buffered formalin, paraffin-embedded, and 5 μm sections were prepared. Standard histology and immunostainings are described in Supplementary Materials and Methods.

Quantification of hepatocyte reactive oxygen species (ROS)

Frozen liver sections (10 μm) from wt and FL-N/35 mice were assessed for the presence of ROS, as previously described [4]. The percentage of ROS-specific hepatocytes was calculated, and normalized to that in wt controls. In all cases (CCl₄-treated and untreated mice), only large cells with round nuclei present within the parenchyma were considered as hepatocytes.

RNA isolation and quantitative real-time PCR analysis

Total RNA and complementary DNA were purified and synthesized, respectively, as previously described [6]. Quantitative PCR was performed with an Applied Biosystems 7300 Thermal Cycler using Taqman reagents (Applied Biosystems), as described in Supplementary Materials and Methods.

Measurement of CCL5 protein in liver tissues

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4 CCL5 protein was quantified in frozen liver tissues according to the procedure
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6 described in Supplementary Materials and Methods.
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10 11 ***Image analysis*** 12 13

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16 Tissue sections were photographed using a Zeiss Axioskop 40 microscope in
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18 conjunction with a Zeiss MRc5 Axiocam and Axiovision LE software (Zeiss, Jena,
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20 Germany). Image analyses were performed as described in Supplementary Materials
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22 and Methods.
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26 27 28 ***Statistical analyses*** 29 30 31

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33 Statistical analyses were performed using Prism software (GraphPad Software
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35 Inc., La Jolla, CA). Results are expressed as mean \pm standard error of the mean (SEM).
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37 Box-and-whisker graphs are used; the line in the middle is the median, the box extends
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39 from the 25th to the 75th percentile, and the whiskers extend to the lowest and highest
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41 values. To determine the statistical significance of the data, Mann-Whitney non-
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43 parametrical tests were performed. Pearson's correlation coefficient was used to
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45 determine correlations between continuous normally distributed variables. All *P* values
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47 were two-tailed, with *P* values less than 0.05 considered as significant.
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RESULTS

Hepatic expression of the full-length HCV ORF enhances liver fibrosis induced by CCl₄ in HCV transgenic mice

The extent of extracellular matrix (ECM) deposition occurring within the liver of wt and FL-N/35 mice chronically exposed to CCl₄ was determined by picosirius red staining of formalin-fixed, paraffin-embedded liver sections. As shown in Fig. 1A and 1B, ECM deposition was observed only in normal anatomical structures such as portal tracts in untreated FL-N/35 and wt mice. Thus, in the absence of an exogenous fibrogenic stimulus, expression of the HCV proteins does not *per se* stimulate ECM deposition at a level detectable using picosirius staining.

In CCl₄-treated wt and FL-N/35 animals, picosirius red staining demonstrated established fibrosis with distinctive septa-like morphologies (Fig. 1A and 1B). In both transgenic and nontransgenic animals, the quantity of ECM was significantly greater than in the corresponding untreated animals (Fig. 1B). In CCl₄-treated mice, the quantity of ECM was significantly greater in FL-N/35 than in wt animals (median collagen deposition: 1.7, range 1.0-2.8 vs 1.2, range, 0.9-2.4; $P=0.0039$) (Fig. 1B).

As differences in the expression of cytochrome P450 2E1 (CYP2E1), the enzyme responsible for bioactivation of CCl₄, could be responsible for the observed difference [30], we quantified CYP2E1 expression by western blotting in HCV transgenic and wt animals. No difference was found between the two groups (Supplementary Fig. S1).

Overall, these results suggest that intrahepatic expression of the HCV proteins *in vivo* potentiates the fibrogenic effect of the exogenous agent CCl₄.

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7 ***Increased liver fibrosis induced by CCl₄ in transgenic mice is not due to a***
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9 ***higher degree of necroinflammation in the presence of HCV proteins***
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14 We examined the role of HCV protein expression in liver necroinflammation in the
15 presence and absence of CCl₄. Hematoxylin-eosin staining of liver tissues showed
16 normal hepatic architecture in wt and FL-N/35 mice that did not receive CCl₄
17 (Supplementary Fig. S2). In contrast, CCl₄-treated animals showed remnants of
18 degenerated and ballooned/necrotic hepatocytes, equivalently present in wt and FL-
19 N/35 mice. Mild mononuclear cell infiltration in the same areas was also evenly present
20 in these animals.
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31 Expression of F4/80 mRNA, a marker of resident and recruited macrophages, did
32 not differ between untreated wt and FL-N/35 animals. F4/80 mRNA expression was
33 induced to the same degree in both transgenic and non-transgenic mice after chronic
34 CCl₄ administration (Supplementary Fig. S3). Immunostaining of liver tissues with the
35 pan-T lymphocyte marker CD3 revealed similar degrees of T lymphocyte infiltration in wt
36 and FL-N/35 mice exposed to CCl₄, with CD3-positive cells present along hepatic septa
37 (Supplementary Fig. S4).
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48 The chemokine CCL5 is secreted by T-cells and macrophages and could play a
49 role in fibrosis by stimulating hepatic stellate cells [31]. CCL5 expression did not differ
50 between wt and FL-N/35 animals, both in the presence and absence of CCl₄
51 (Supplementary Figs. S5 and S6).
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57 Taken together, these results suggest that enhanced fibrosis in HCV-transgenic
58 mice exposed to CCl₄ is not mediated by an increased necroinflammation.
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7 **Increased liver fibrosis induced by CCl₄ in transgenic mice is associated**
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9 **with alterations in the expression of genes involved in ECM remodeling**

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14 We measured the hepatic expression of a number of ECM remodeling genes,
15 including genes coding for ECM components (collagens), proteins involved in ECM
16 production/degradation (matrix metalloproteinases [MMP]) and regulators of MMPs
17 (tissue inhibitors of metalloproteinase [TIMP]) in the different groups of mice. As shown
18 in Fig. 2, in the absence of an exogenous fibrogenic stimulus, the expression of HCV
19 proteins in hepatocytes was not associated with higher levels of expression of these
20 genes. In CCl₄-treated wt animals, expression of all of the tested mRNA levels was
21 significantly modified compared to the untreated mice, but in various directions (Fig. 2).
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23 Among the different collagen genes tested, only collagen 1 α 1 expression was notably
24 enhanced in FL-N/35 compared to wt CCl₄-treated mice, although the results did not
25 reach statistical significance (p = 0,055). The expression of the other collagens showed
26 no difference (Fig. 2A). MMP7 mRNA levels were significantly lower in HCV transgenic
27 than in non-transgenic CCl₄-treated animals, whereas the expression of MMP13, MMP3
28 and TIMP1 was notably but not significantly lower (p values between 0.052 and 0.055,
29 Fig. 2B and 2C). These results were in keeping with the increase in ECM deposition
30 observed in HCV transgenic animals treated with CCl₄.

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55 ***Liver fibrosis is associated with decreased hepatocyte proliferation in HCV***
56 ***transgenic mice chronically exposed to CCl₄***
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7 Since hepatic fibrosis in HCV-infected patients has been associated with
8 increased hepatocyte replicative arrest [29], we next examined the extent of hepatocyte
9 proliferation in wt and FL-N/35 mice in the presence and absence of CCl₄. Hepatocyte
10 proliferation was evaluated by immunohistochemistry for Ki67, a nuclear antigen that is
11 expressed in G1, S, G2 and M phases of the cell cycle, but absent in G0 or quiescent
12 phase. In the absence of chronic CCl₄ exposure, a very low proportion of proliferative
13 hepatocytes was observed in both wt and FL-N/35 mice, with no differences between
14 these two groups, suggesting low-level basal renewal of the mature hepatocyte pool in
15 the parenchyma (Fig. 3A and 3B).
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28 As a consequence of chronic exposure to CCl₄, hepatocyte proliferation was
29 significantly increased in both wt and FL-N/35 mice compared to non-exposed animals
30 (Fig. 3A and 3B). As shown in Fig. 3B, hepatocyte proliferation was significantly lower in
31 CCl₄-exposed FL-N/35 than in wt mice (median positive hepatocytes, 25.1 %, range 7.1-
32 65.7 %, vs 30.3 %, range 5.9-49.2 %, respectively; $P=0.002$). No difference in the
33 distribution of proliferating hepatocytes within the parenchyma was observed between
34 treated wt and FL-N/35 animals.
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45 In HCV-transgenic mice exposed to CCl₄, a significant inverse relationship
46 between the number of Ki67 positive hepatocytes and the extent of ECM deposition was
47 observed ($r=0.893$, $P=0.012$; Fig. 3C). In contrast, no correlation was found in CCl₄-
48 exposed wt mice ($r=0.1022$, $P=0.81$; Fig. 3D).
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55 In concert, these results suggest that fibrosis induced by CCl₄ exposure in the
56 context of HCV protein expression is concomitant with a lowered capability of
57 hepatocytes to regenerate.
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7 ***A prominent ductular reaction is present in HCV transgenic mice***
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9 ***chronically exposed to CCl₄***
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14 Since decreased hepatocyte proliferation has been linked with the development
15 of a ductular reaction in several hepatic disorders, in particular in HCV infection [29, 32],
16 we next studied the ductular reaction in both wt and FL-N/35 mice by immunochemistry
17 for cytokeratin 19 (CK19), a common bile duct, ductular epithelium and HPC marker in
18 murine tissues. CK19-positive staining was limited to bile duct cholangiocytes in
19 untreated wt and FL-N/35 mice, with no differences between the two groups (Fig. 4A
20 and 4B).
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31 Exposure of wt animals to CCl₄ did not modify the quantity and distribution of
32 CK19-positive biliary epithelium (Fig. 4A and 4B). In contrast, HCV transgenic animals
33 chronically exposed to CCl₄ presented a prominent positive staining for CK19,
34 distributed at the periphery of the portal areas, in chords, clumps and rings, quite distinct
35 from the anatomical bile ducts (Fig. 4A). The area occupied by CK19-positive biliary
36 epithelium, comprising both anatomical bile ducts and the ductular reaction, was
37 increased nearly 3-fold compared with CCl₄-exposed wt mice (0.59% and 0.18%
38 respectively, $P=0.0016$; Fig. 4B).
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50 Altogether, these results suggest that fibrosis induced by CCl₄ exposure in the
51 context of HCV protein expression is concomitant with a ductular reaction, which is
52 absent in similarly treated wt animals.
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4 ***The ductular reaction is associated with expansion of the HPC population in***
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6 ***HCV transgenic mice chronically exposed to CCl₄***
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11 Since hepatic fibrosis in HCV-infected patients has been associated with
12 increased HPC expansion [29], we assessed whether the increased ductular reaction
13 was associated with expansion of the HPC population in HCV transgenic animals
14 exposed to CCl₄. HPCs were distinguished from cholangiocytes as being CK19-positive
15 cells distal to sites of ductular reaction and isolated within the parenchyma, without the
16 cord-like arrangement of the ductules. In the absence of CCl₄, a few HPCs were visible
17 in the livers from either wt or FL-N/35 mice (Fig. 4A and 4C). After chronic CCl₄
18 treatment, a non-significant trend towards a higher number of isolated CK19-positive
19 cells than in the untreated animals was observed in wt mice (Fig. 4A and 4C). In CCl₄-
20 treated FL-N/35 mice, a statistically significant increase in CK19-positive cells was
21 observed when compared to CCl₄-treated wt mice ($P=0.0016$; Fig. 4C).
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38 We observed significant correlations between the number of HPCs per portal tract
39 and the extent of ECM deposition ($r=0.71$, $P=0.0062$; Fig. 4D), and between the number
40 of HPCs and the surface of the ductular reaction ($r=0.7984$, $P=0.0011$; Fig. 4E), which
41 were independent of HCV protein expression. However, FL-N/35 animals displayed
42 increased ECM deposition, an increased number of HPCs per portal tract, and a larger
43 ductular reaction when compared with wt animals, suggesting that the viral proteins may
44 exacerbate these phenomena.
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55 Together, these data indicate that the increased ductular reaction observed in
56 HCV transgenic mice is associated with expansion of the HPC population.
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4 ***The expansion of the HPC population leads to normal liver reconstitution in***
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6 ***HCV transgenic mice chronically exposed to CCl₄***
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11 As shown in Table 1, despite the decreased hepatocyte proliferation in FL-N/35
12 transgenic mice chronically exposed to CCl₄ shown in Figs. 3A and 3B, no difference
13 was observed in body or liver weights between olive oil- and CCl₄-treated animals in
14 both wt and FL-N/35 groups, suggesting that liver tissue reconstitution is not
15 substantially impaired in HCV transgenic mice.
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26 ***Hepatocyte cell cycle progression is impaired after acute liver injury in HCV***
27 ***transgenic mice***
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33 In order to assess whether HCV protein expression impairs liver regeneration,
34 hepatocyte proliferation was measured in both wt and FL-N/35 mice during the course of
35 acute CCl₄-induced liver injury (Fig. 5A). Analysis of BrdU incorporation showed no or
36 very few BrdU-positive cells in wt and HCV transgenic mouse livers before CCl₄ injection
37 (0h), indicating that the majority of liver cells were quiescent in G₀. As shown in Fig. 5A
38 and 5B, both wt and HCV transgenic animals showed a significant increase of BrdU-
39 positive hepatocytes after CCl₄ injection, suggesting entry into the S-phase of the cell
40 cycle. However, this phenomenon was delayed in HCV transgenic animals, with a peak
41 of BrdU incorporation reached at 48 hours post-injection, compared to 40 hours post-
42 injection in wt animals (Fig. 5B). These findings indicate that HCV protein expression is
43 associated with a delay in early cell cycle progression after acute liver injury.
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4 **ROS production is enhanced in HCV transgenic mice treated or not with**
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6 **CCl₄**
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11 Since oxidative stress has been reported to modulate the fibrogenic response and
12 elevated ROS production is frequently observed in HCV infected cells [33, 34], we
13 measured ROS levels in the livers of wt and FL-N/35 mice. Using dihydroethidium (DHE)
14 as a marker for intracellular ROS, we found that hepatocyte-specific ROS levels were
15 significantly higher in frozen liver sections from HCV transgenic mice compared to their
16 wt littermates in the absence of CCl₄ treatment (Fig. 6A, upper panels and Fig. 6B). In
17 CCl₄-treated animals, ROS levels were significantly higher than in untreated animals in
18 both the wt and FL-N/35 groups (Fig. 6A, lower panels), principally due to infiltrating
19 cells. However, when ROS production was measured only in hepatocytes, the levels
20 were significantly higher in CCl₄-exposed FL-N/35 mice than in their wt counterpart
21 animals (Fig. 6A, lower panels and Fig. 6B). These results show that intrahepatic
22 expression of the HCV proteins is associated with enhanced baseline ROS levels and
23 that this phenomenon is amplified by CCl₄ treatment.
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45 **DISCUSSION**
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50 Several *in vitro* reports have suggested that HCV proteins can directly induce
51 fibrogenetic processes. For example, it has been shown that the culture medium of
52 Huh7 cells harboring the HCV subgenomic replicon is able to activate *in vitro*-cultured
53 HSCs, demonstrating that virus production is not a pre-requisite for such activation [35].
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55 Moreover, the core and nonstructural 5A (NS5A) proteins have been suggested to play
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4 a direct role in the alteration of the hepatocyte metabolism, partly *via* mitochondrial
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6 deregulation which generates ROS, strong inducers of pro-fibrogenic cytokines within
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8 the liver [1, 36, 37].
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11 The mouse model used in this study is ideal to assess the effect of HCV protein
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13 expression on a liver phenomenon. Indeed, these mice specifically express the different
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15 HCV proteins in the liver; however, they are unable to reproduce the complete HCV life
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17 cycle, because the transgene is composed of a cDNA corresponding to the full-length
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19 polyprotein coding region, whereas the 5' and 3' untranslated sequences of the viral
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21 genome necessary for replication of viral RNA are lacking [1].
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25 Our findings suggest that HCV protein expression *per se* is not able to induce
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27 fibrosis at a level detectable by common histological and molecular techniques.
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29 Nevertheless, we demonstrate for the first time that hepatic expression of the full-length
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31 HCV ORF *in vivo* is able to enhance fibrosis induced by another fibrogenic agent, a
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33 context similar to chronic human infection where a number of fibrogenic triggers are
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35 present in the liver. Importantly, this effect is independent of the degree of local
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37 inflammation. Our finding that ROS production is significantly increased in HCV
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39 transgenic hepatocytes, both in the absence and presence of CCl₄ treatment, could at
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41 least partly explain that HCV transgenic mice are more susceptible to the development
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43 of liver fibrosis when they are exposed to a 'second hit' by a fibrogenic agent.
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51 We show that enhanced liver fibrosis in HCV transgenic animals is associated
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53 with a deregulation of the expression of several matrix remodeling genes. This includes
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55 down-regulation of the expression of MMP13, a protease that specifically degrades
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57 collagen 1, TIMP1, a protein that inhibits MMPs active forms [38], and MMP3, an
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59 activator of pro-MMPs. Thus, HCV transgenic animals seem to be less able to degrade
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4 collagen 1, thereby favoring ECM deposition. In addition, the expression of MMP7, a
5 protease implicated in nerve growth factor-dependent myofibroblast survival [39], was
6 reduced in CCl₄-treated HCV mice, whereas a similar trend was found in untreated
7 transgenic animals, suggesting that HCV protein expression could protect hepatic
8 myofibroblasts from apoptosis, thus prolonging fibrosis signals.

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11 In our study, the enhancement of fibrosis induced by another fibrogenic agent in
12 transgenic mice expressing the full-length HCV ORF is accompanied by reduced
13 hepatocyte proliferation, which correlates with the stage of fibrosis. The replacement of
14 hepatocytes lost from normal hepatic parenchyma is known to occur through replication
15 of mature hepatocytes [16]. Inhibition of hepatocyte replication by drugs [16], alcohol
16 [40], steatosis [15] or viral infection [14, 29] promotes the activation of a secondary
17 replicative pathway involving bipotential HPCs [16]. The activation of HPCs
18 subsequently leads to the production of both hepatocytes and cholangiocytes, and the
19 onset of a ductular reaction. The results presented here show that hepatocytes
20 expressing the full HCV ORF exhibit defective replication after treatment with CCl₄. Our
21 acute liver injury experiments suggest that this effect is, at least in part, due to a delayed
22 entry of hepatocytes into the S-phase of the cell cycle.

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25 The defective replication of HCV-expressing hepatocytes leads to the activation
26 of the HPC pathway and subsequent amplification of the HPC population. In keeping
27 with this hypothesis, we observed reduced proliferation of mature hepatocytes together
28 with a marked increase in the number of activated HPCs and a prominent ductular
29 reaction in CCl₄-treated FL-N/35 mice. A strong relationship between hepatic fibrosis
30 severity and the intensity of the ductular reaction has been reported in both rodents and
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4 humans [29, 32, 41, 42]. A similar relationship is shown here in the FL-N/35 mouse
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6 strain in the context of HCV protein-induced liver fibrosis [29].
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9 In summary, we have shown that transgenic mice expressing the full-length HCV
10 ORF develop more severe hepatic fibrosis after chronic CCl₄ exposure than
11 nontransgenic animals. This effect is not linked to local inflammation alterations induced
12 by HCV proteins. Like during chronic HCV infection in humans, enhanced fibrosis in
13 HCV transgenic mice is associated with a pronounced ductular reaction, HPC activation
14 and a concomitant reduction in hepatocyte proliferation that appears to be in part due to
15 a delayed entry of hepatocytes into the S-phase of the cell cycle. In addition, HCV-
16 related fibrosis is associated with deregulation of the expression of several matrix
17 remodeling genes and enhanced ROS production that likely participate in the fibrogenic
18 process. Overall, our findings in HCV transgenic mice show that expression of the HCV
19 proteins in hepatocytes contributes to the development of hepatic fibrosis in the
20 presence of other fibrogenic agents, a situation close to human infection.
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FIGURE LEGENDS

Fig 1. Histological analysis of liver fibrosis in wt and FL-N/35 mice in the absence or presence of chronic CCl₄ exposure. (A) Representative images of picrosirius red-staining of liver sections from olive-oil and CCl₄-injected wt and FL-N/35 animals. Scale bars: 100 μm. **(B)** Quantification of picrosirius red-staining from images from various lobes of each liver (6 to 10 images per liver). NS: not significant.

Fig. 2. mRNA level quantification of genes involved in ECM remodeling in the livers of wt and FL-N/35 mice in the absence or presence of chronic CCl₄ exposure. mRNA levels of collagens (A), MMPs (B) and TIMPs (C) were measured by means of qRT-PCR. mRNA levels were normalized to the expression of actin; values are expressed as the percentage of wt animals treated with olive oil (OO).

Fig. 3. Hepatocyte proliferation in wt and FL-N/35 mice in the absence or presence of chronic CCl₄ exposure. (A) Representative images of Ki67 immunostaining of liver sections from olive-oil and CCl₄-treated wt and FL-N/35 animals. Positive Ki67 nuclei are indicated with arrows. Scale bars: 100 μm. **(B)** Quantification of Ki67-positive hepatocytes from various lobes of each liver. Data are expressed as a percentage of total hepatocytes per field. NS: not significant. **(C)** Relationship between hepatocyte proliferation and the extent of CCl₄-induced ECM deposition in HCV-transgenic mice treated with CCl₄. Data points represent individual animals. Analysis was performed with 9 animals from 2 independent experiments. **(D)** Relationship between hepatocyte

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4 proliferation and the extent of CCl₄-induced ECM deposition in wt mice treated with
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6 CCl₄. Data represents 8 animals from 2 independent experiments.
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11 **Fig. 4. Ductular reaction in the livers of wt and FL-N/35 mice in the absence or**
12 **presence of chronic CCl₄ exposure. (A)** CK19-immunostaining of liver sections from
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14 olive oil- and CCl₄-treated wt and FL-N/35 animals. Open arrows indicate CK19-positive
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16 bile ducts. Black arrows indicate prominent ductules. Arrowheads indicate hepatic
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18 progenitor cells (HPCs). The two lower images show higher magnifications. **(B)**
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20 Quantification of the ductular reaction (CK19-positive bile ducts) in images of CK19
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22 immunostaining of olive oil- and CCl₄-treated animals. **(C)** Quantification of isolated
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24 HPCs (isolated CK19-positive cells nearby portal tracts) in images of CK19
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26 immunostaining of olive oil- and CCl₄-treated animals. **(D)** Relationship between the
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28 number of HPCs and the extent of CCl₄-induced ECM deposition. **(E)** Relationship
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30 between the extent of the ductular reaction and the number of HPCs.
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41 **Fig. 5. Hepatocyte DNA synthesis in wt and FL-N/35 mice after acute liver injury**
42 **caused by high-dose CCl₄ Injection. (A)** Representative photomicrographs of BrdU
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44 immuno-histochemical staining in acutely injured livers from wt and FL-N/35 mice at 0h,
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46 24h, 40h, 48h and 60h post CCl₄ injection. BrdU-positive nuclei are stained in red. Scale
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48 bars = 100 μm. (B) BrdU incorporation at 0h, 24h, 40h, 48h and 60h post CCl₄ injection
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50 in wt and FL-N/35 animals. The number of BrdU-positive hepatocytes was expressed
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52 per μm² of tissue area.
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4 **Fig. 6. Quantification of ROS levels in the livers of wt and FL-N/35 mice in the**
5 **absence or presence of chronic CCl₄ exposure. (A) Frozen 10 μm liver sections of**
6 **olive oil and CCl₄-treated wt and FL-N/35 mice were stained with DHE and DAPI (insert),**
7 **and analyzed by fluorescence microscopy. Representative examples are shown. Scale**
8 **bars = 50 μm. (B) The number of ROS-positive hepatocytes was measured, and is**
9 **shown as the percentage of ROS-positive cells as described in the Material and**
10 **Methods section.**
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Table 1. Age, body and liver weights and ALT/AST levels in wt and FL-N/35 mice chronically exposed or not to CCl₄. Mean±SEM values are shown. NS: not significant.

	Olive oil		p	CCl ₄		p
	wt (n=15)	FL-N/35 (n=15)		wt (n=13)	FL-N/35 (n=17)	
Age (month)	8-10	8-10		8-10	8-10	
Body weight (g)	33.7±0.8	33.9±1.2	NS	30.0±3.5	31.3±3.9	NS
Liver weight (g)	1.5±0.04	1.5±0.09	NS	1.5±0.3	1.4±0.4	NS
ALT (IU/mL)	42±12.6	46±22.8	NS	2570.5±479.5	1654.8±316.9	NS
AST (IU/mL)	63.2±20.7	81.6±32.8	NS	1532.5±319.7	921±170	NS

Figures
Figure 1A

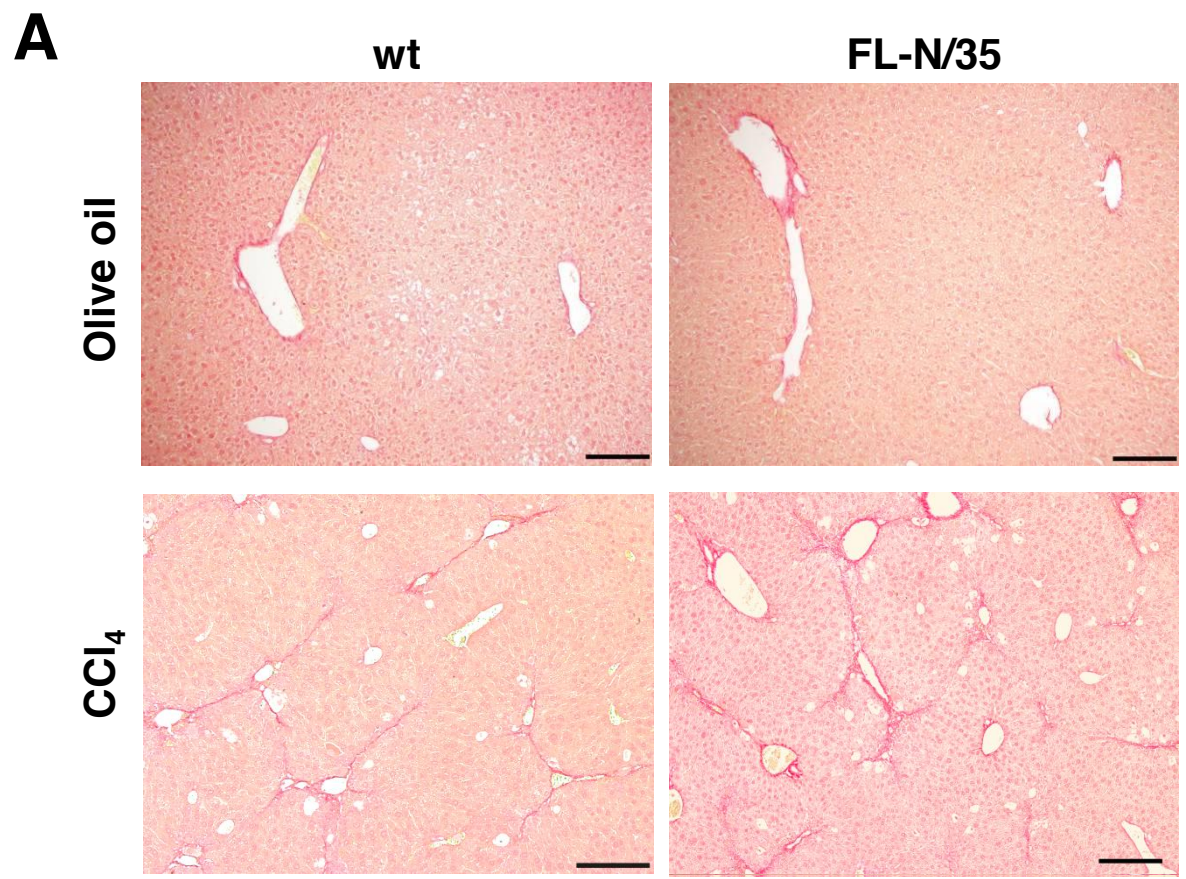


Figure 1B

B

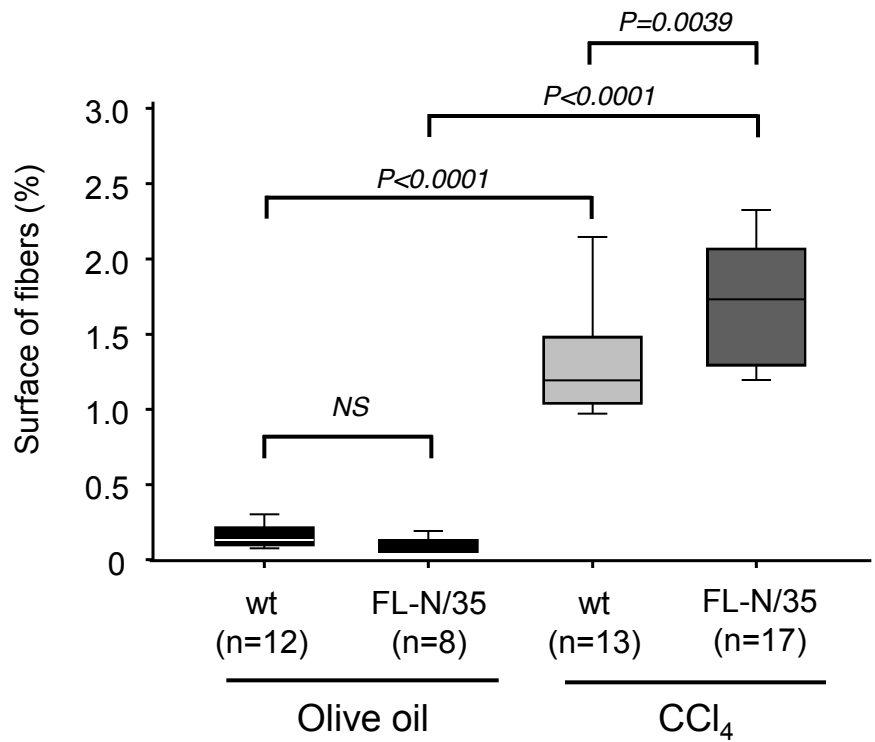


Figure 2A

A

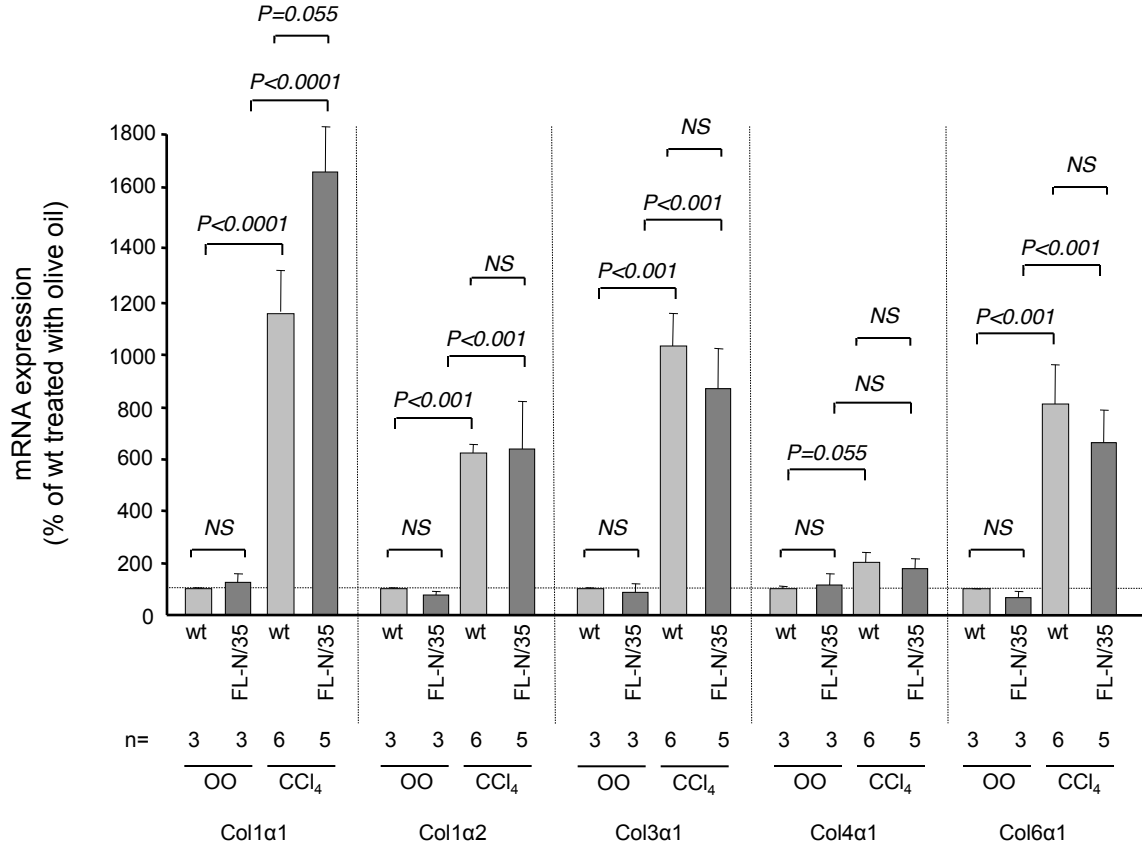


Figure 2B

B

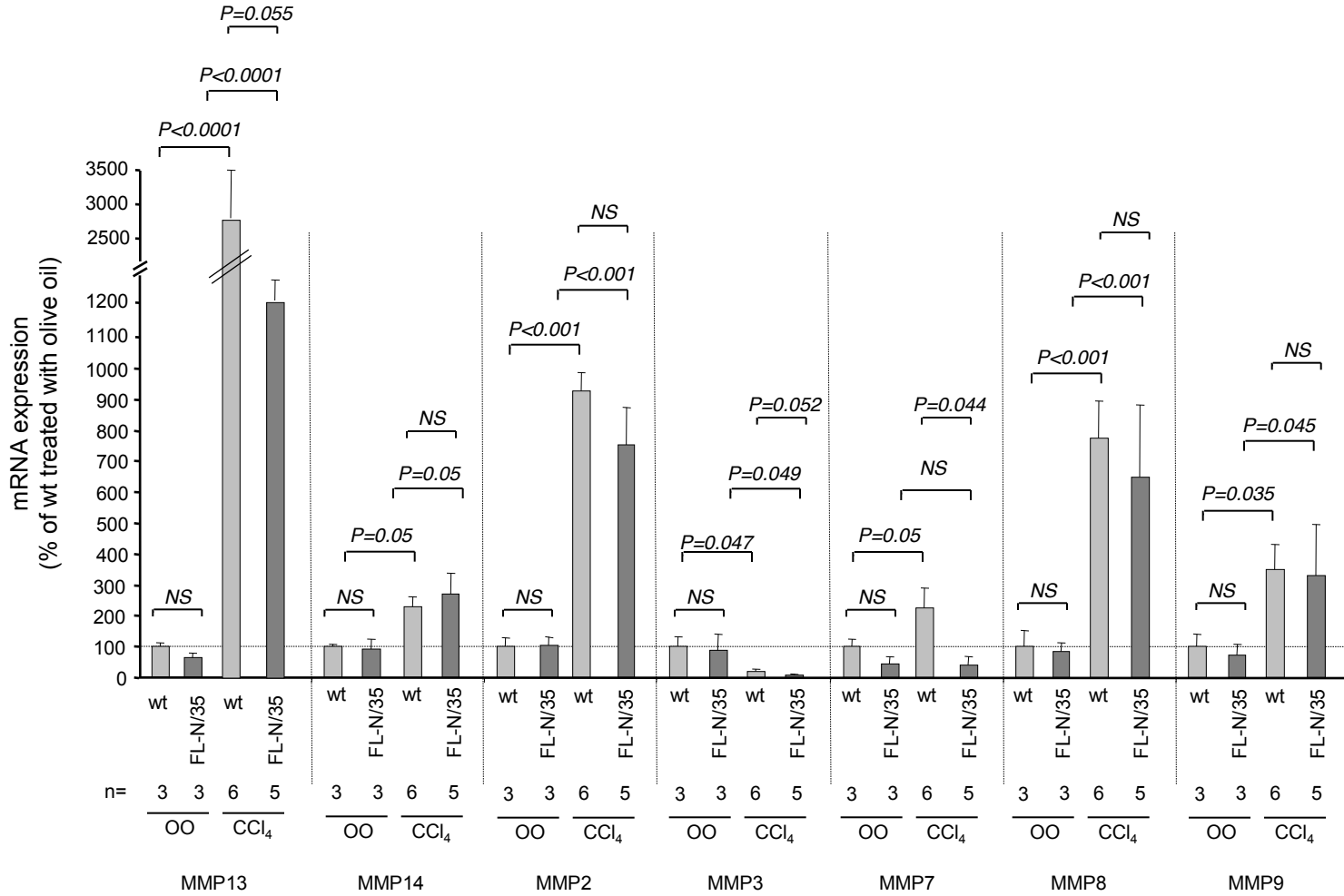


Figure 2C

C

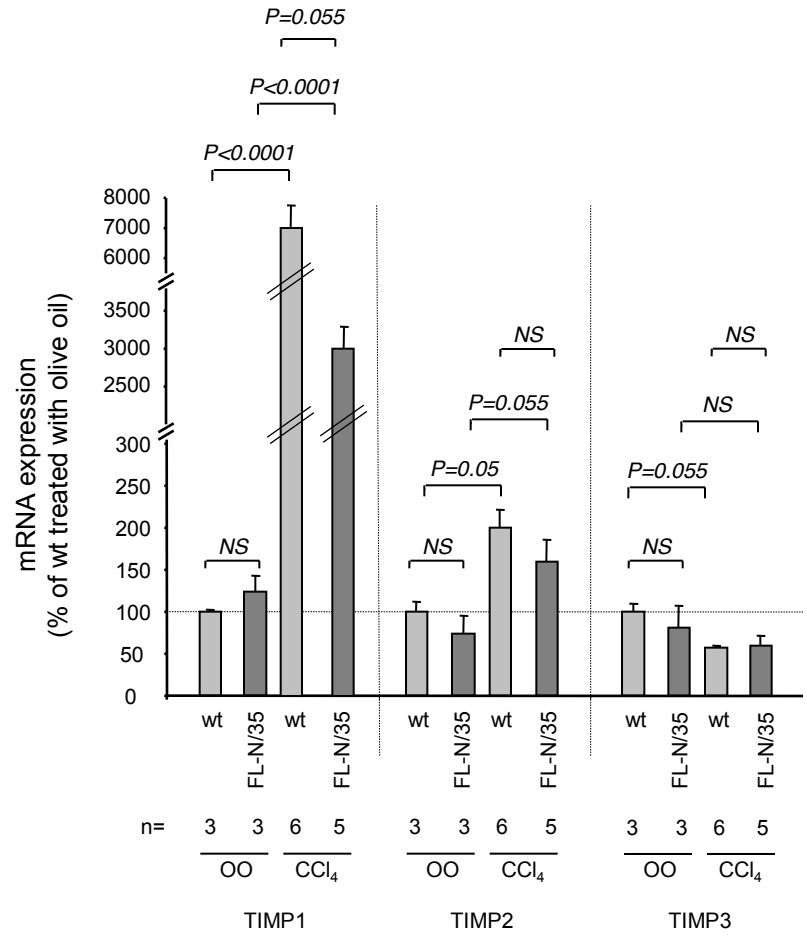


Figure 3A

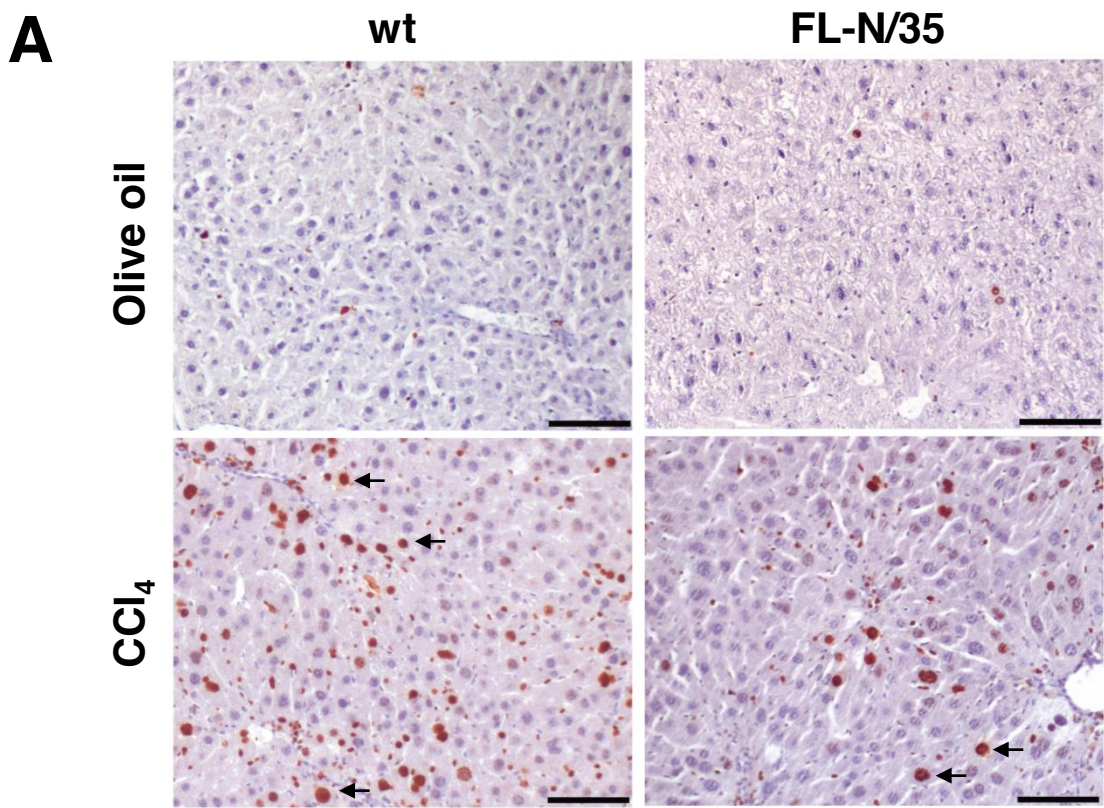


Figure 3B

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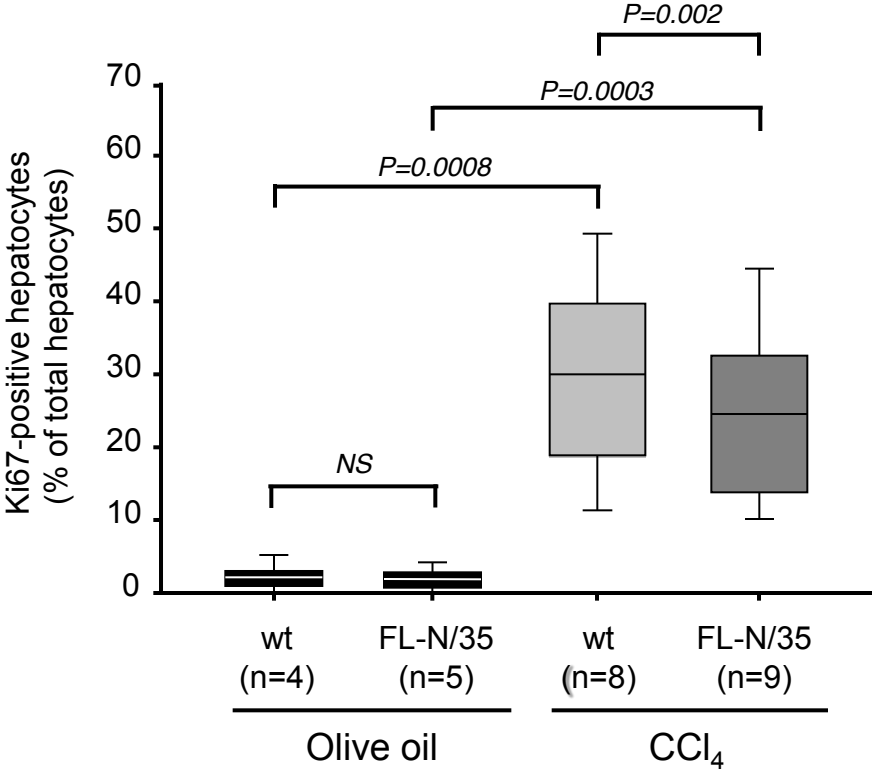


Figure 3C

C

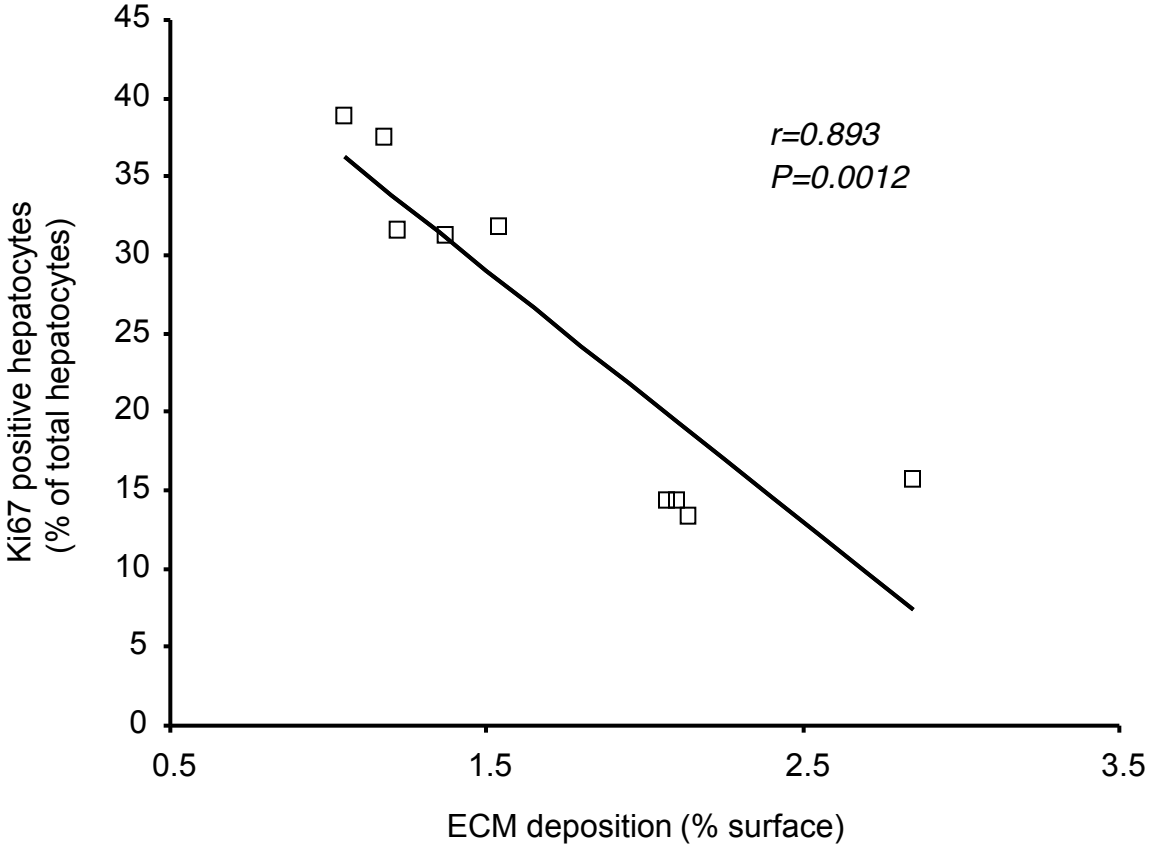


Figure 3D

D

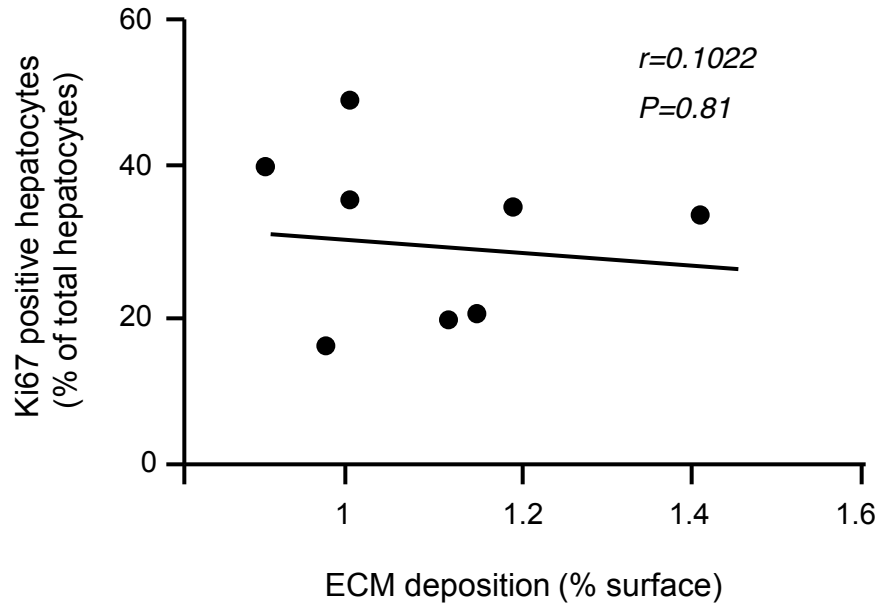


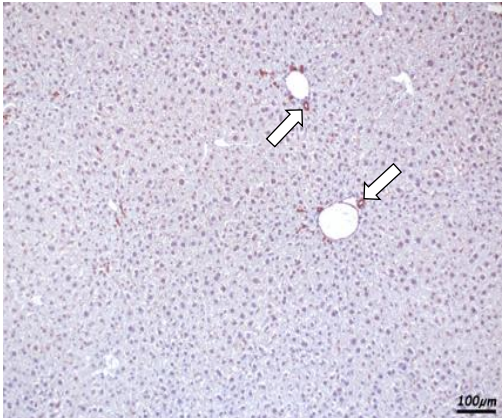
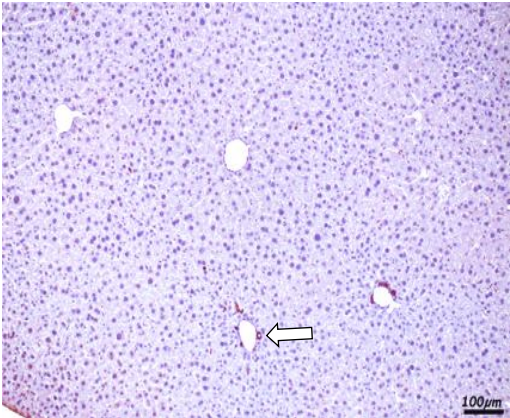
Figure 4A

A

wt

FL-N/35

Olive oil



CCl₄

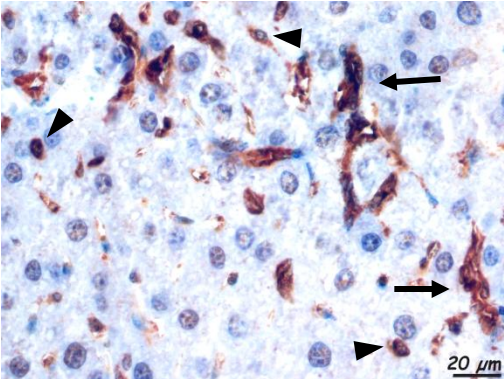
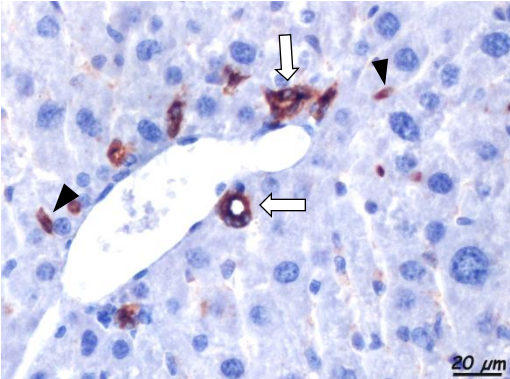
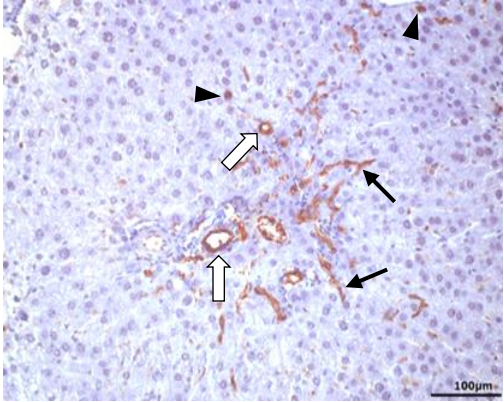
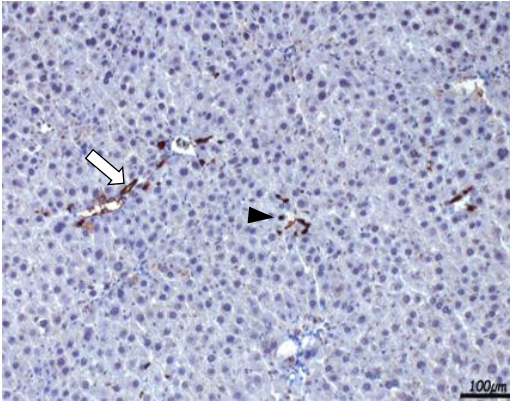


Figure 4B

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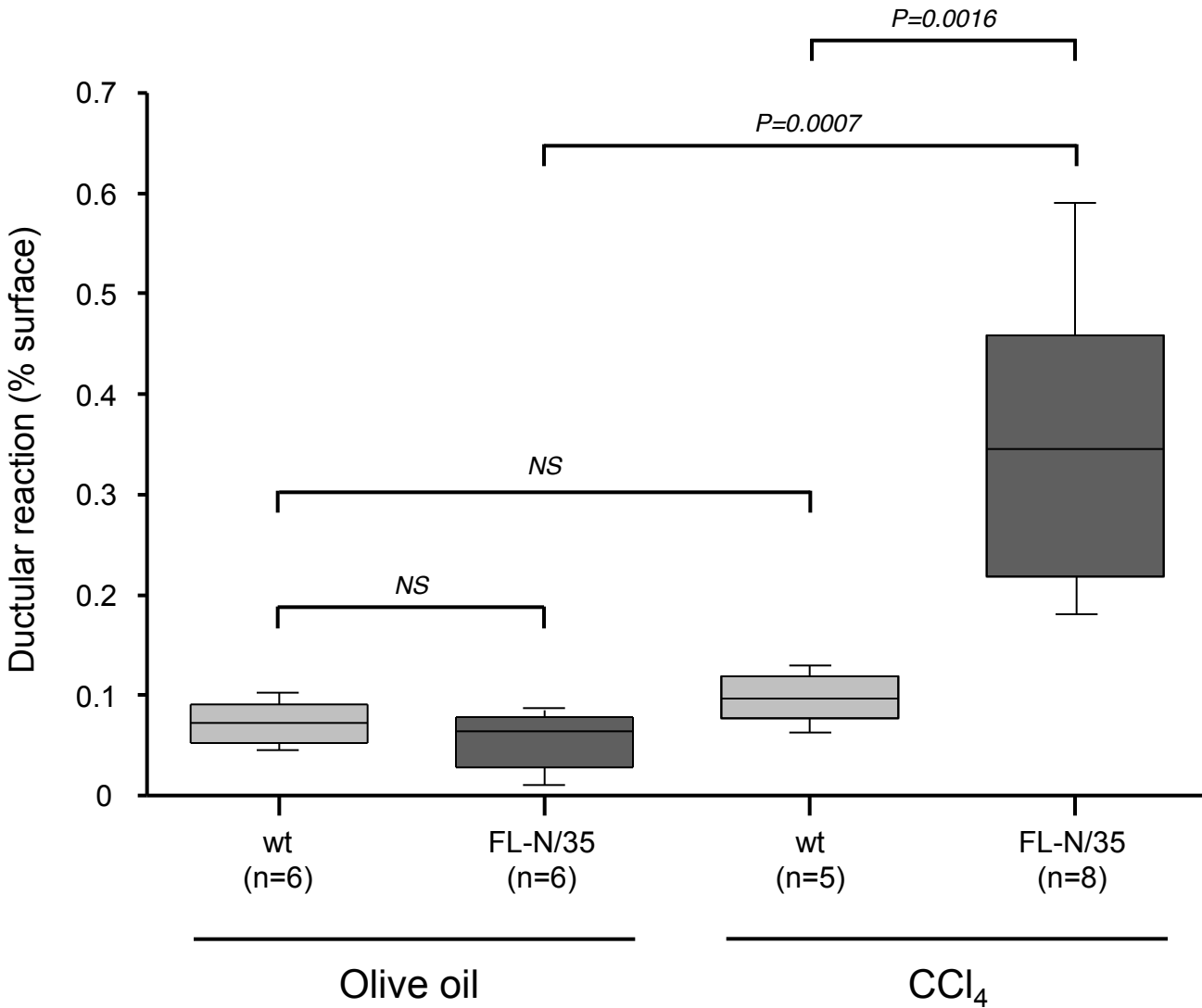


Figure 4C

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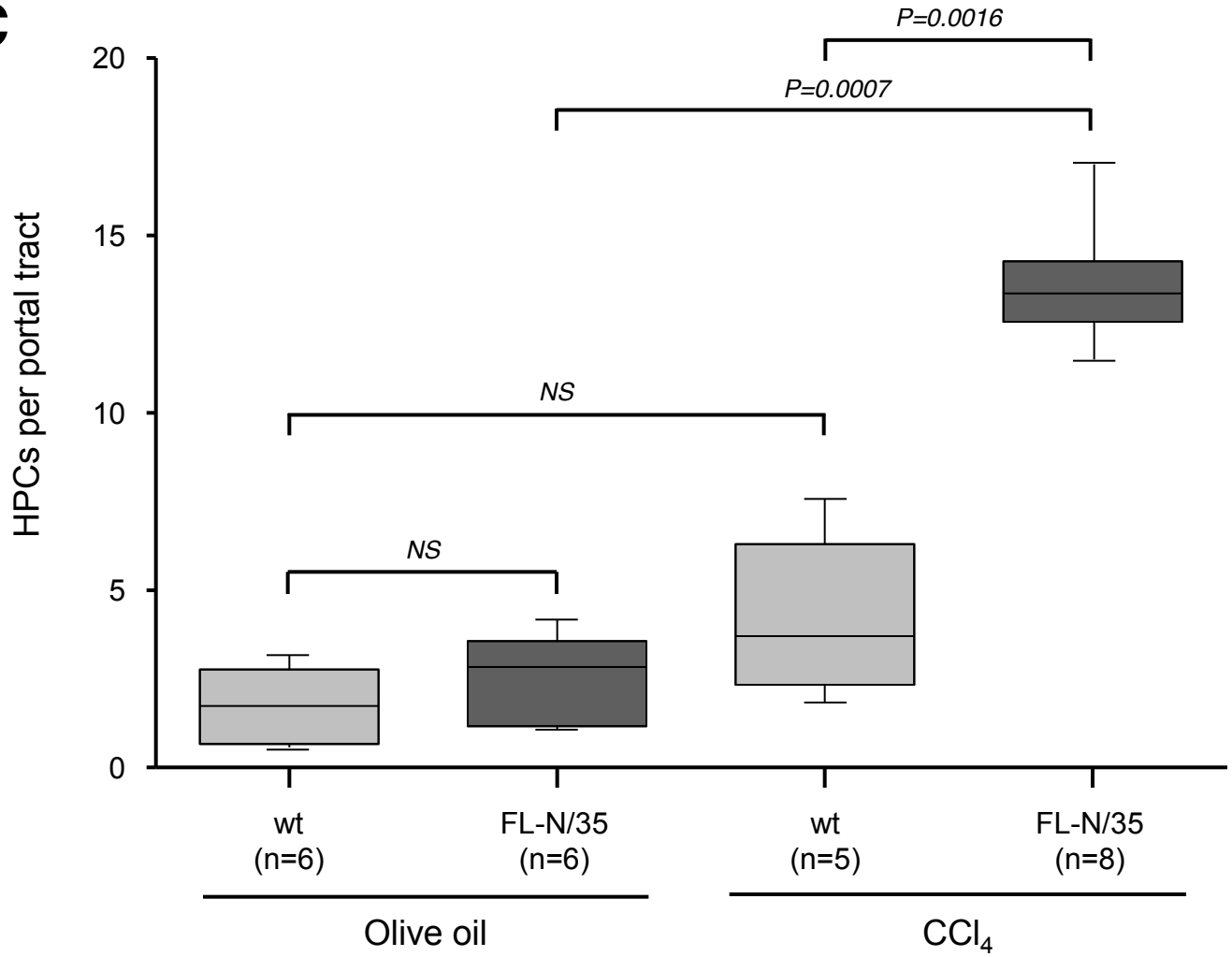


Figure 4D

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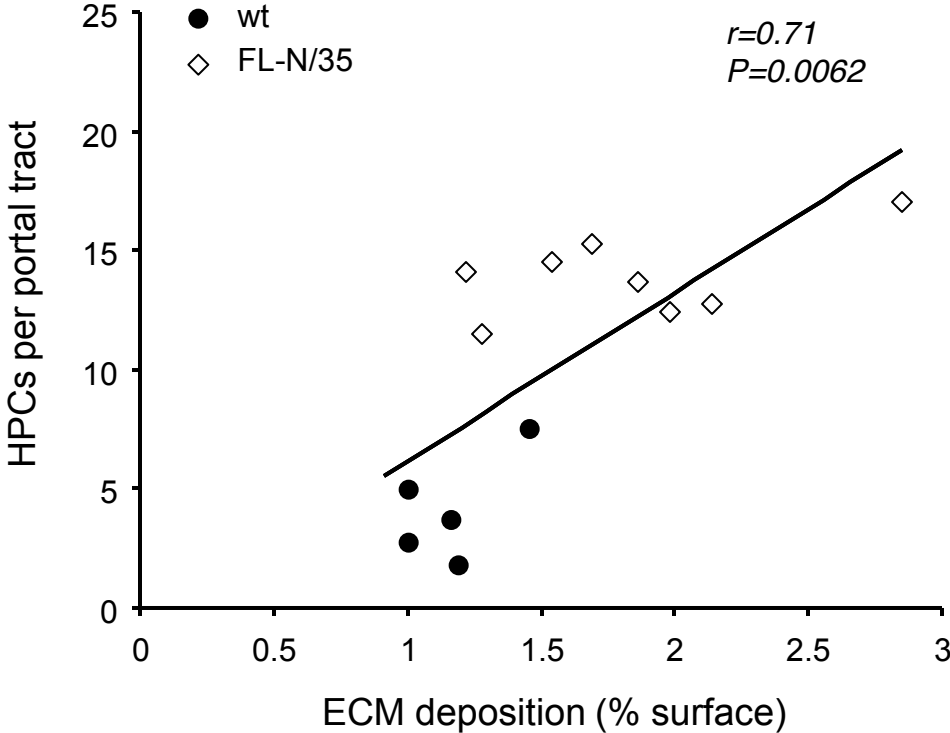


Figure 4E

E

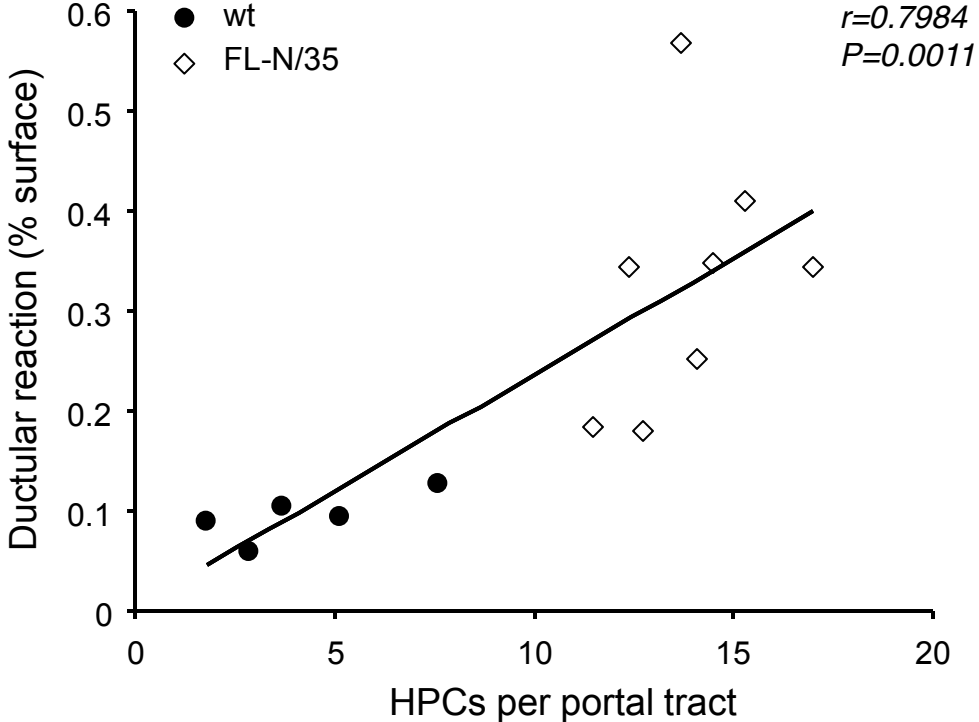


Figure 5A

A

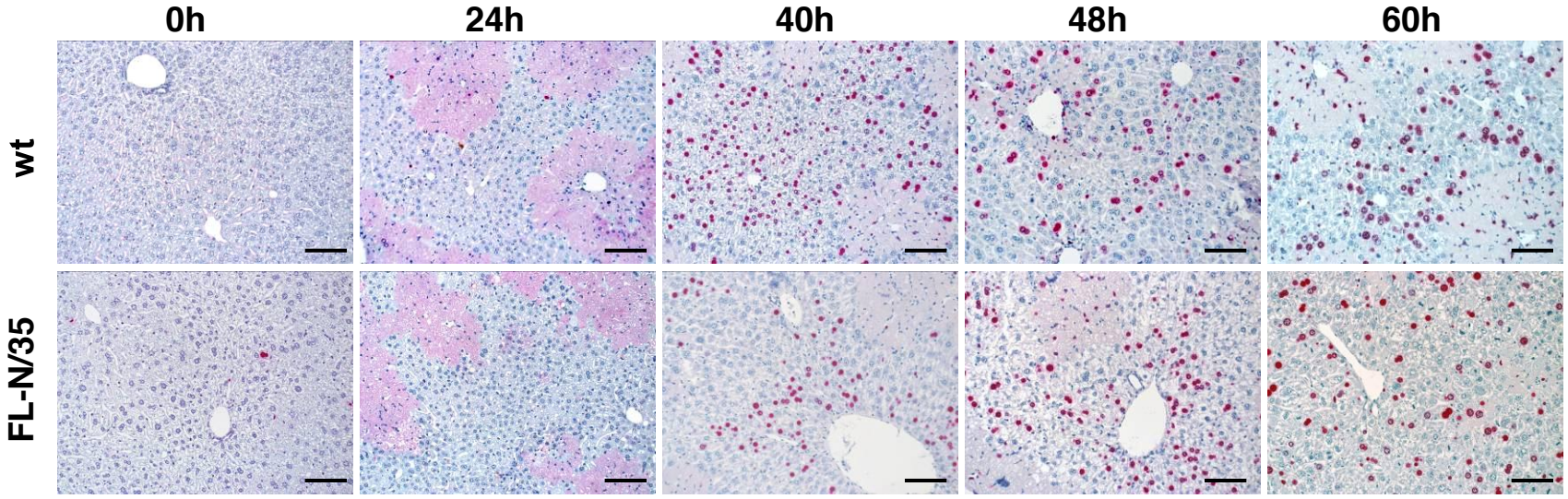


Figure 5B

B

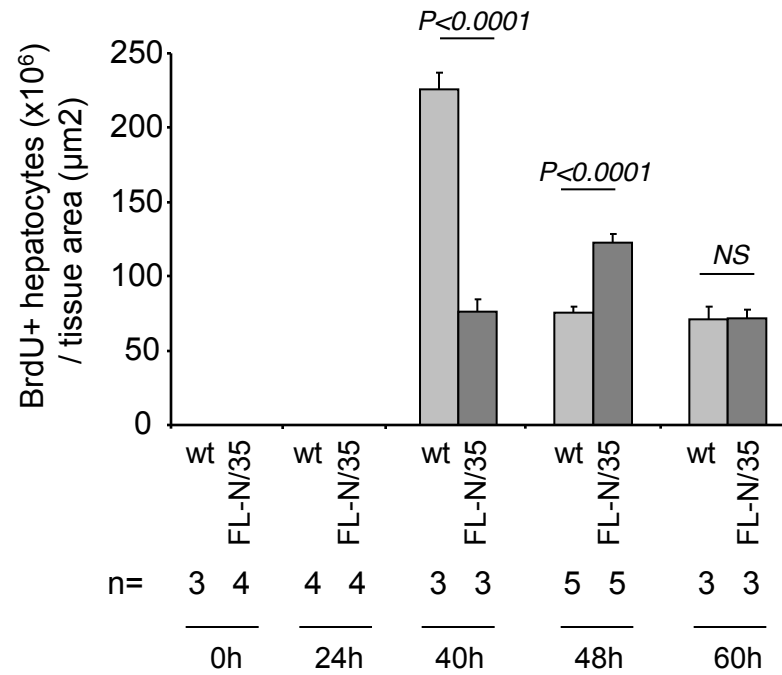


Figure 6A

A

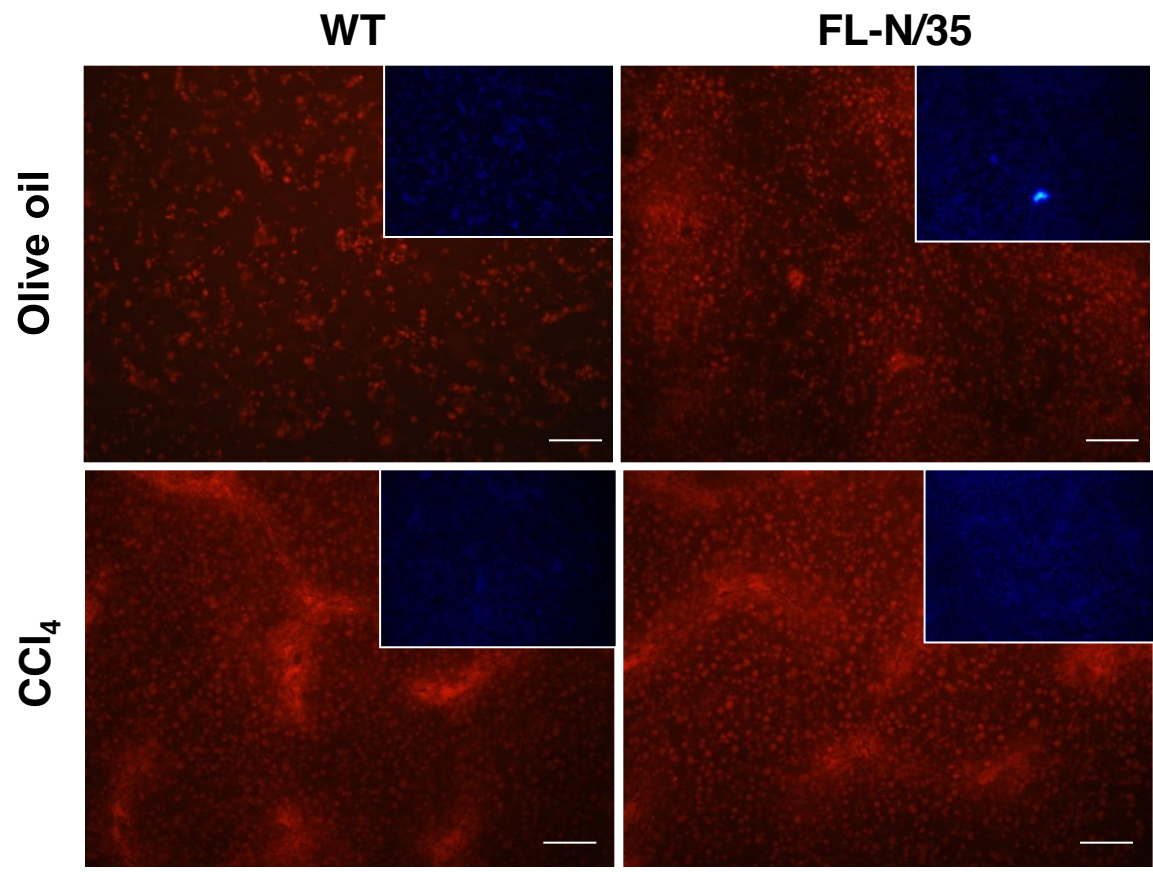
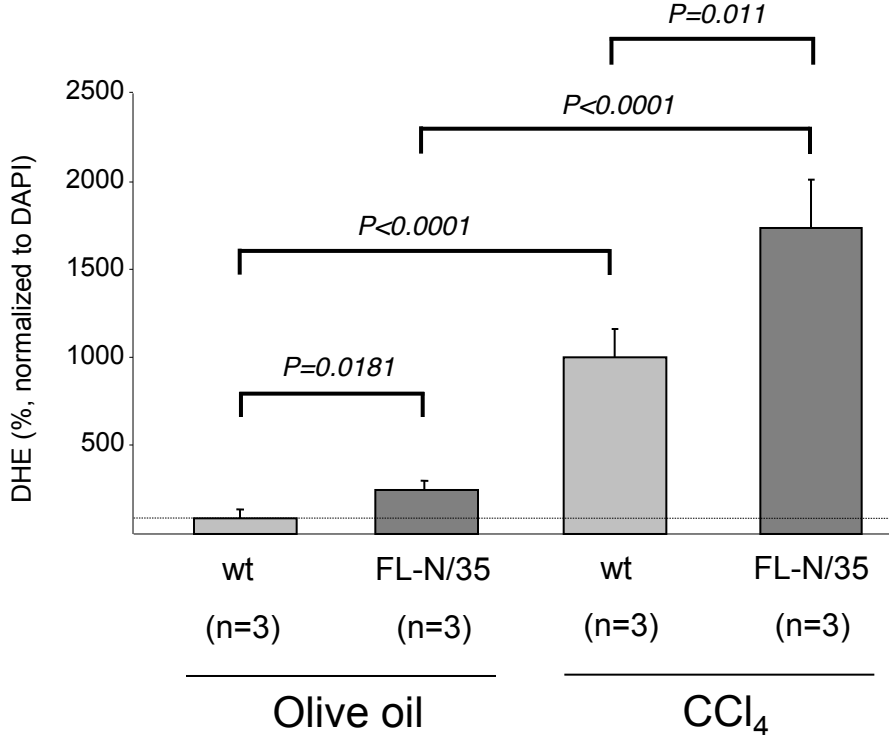


Figure 6B

B



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