SUPPLEMENTARY FIGURES

Dissecting the role of the NADPH Oxidase NOX4 in TGF-beta signaling in hepatocellular carcinoma

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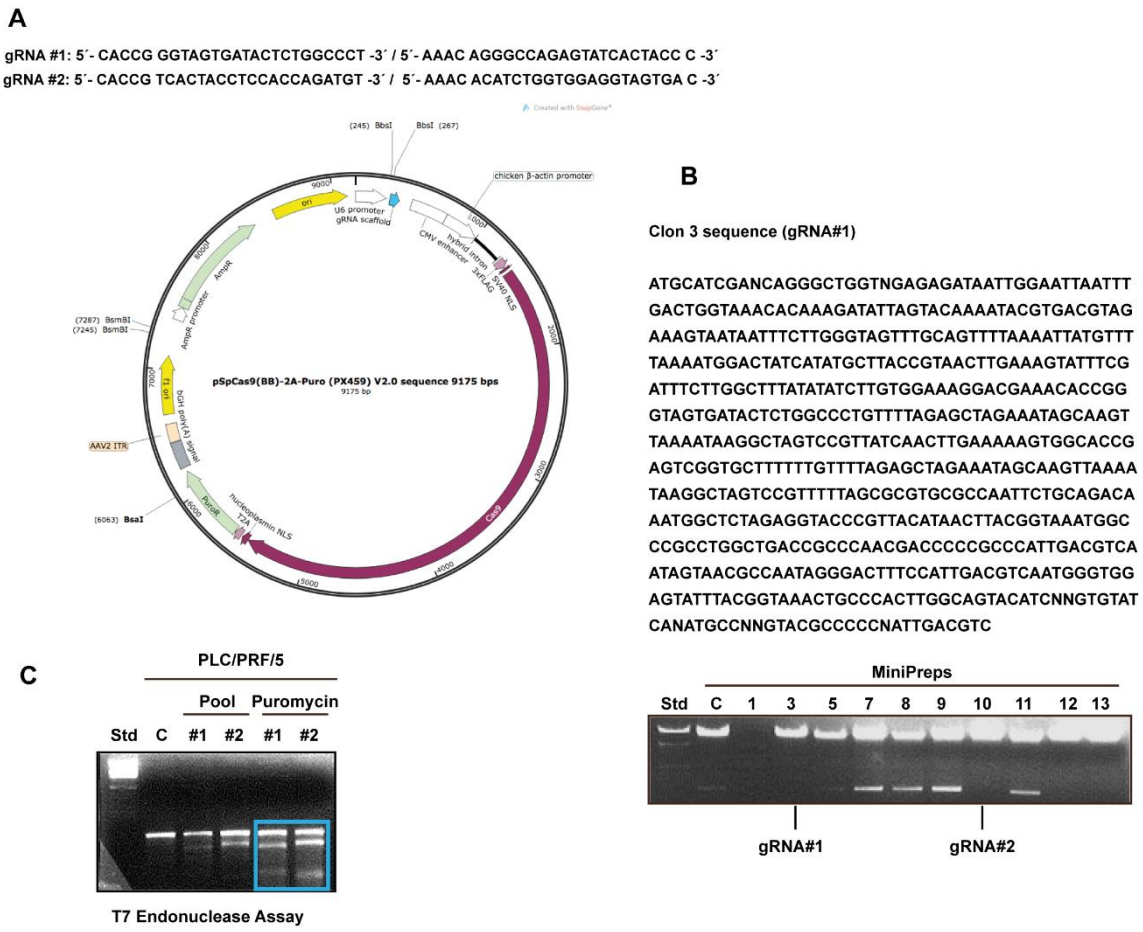
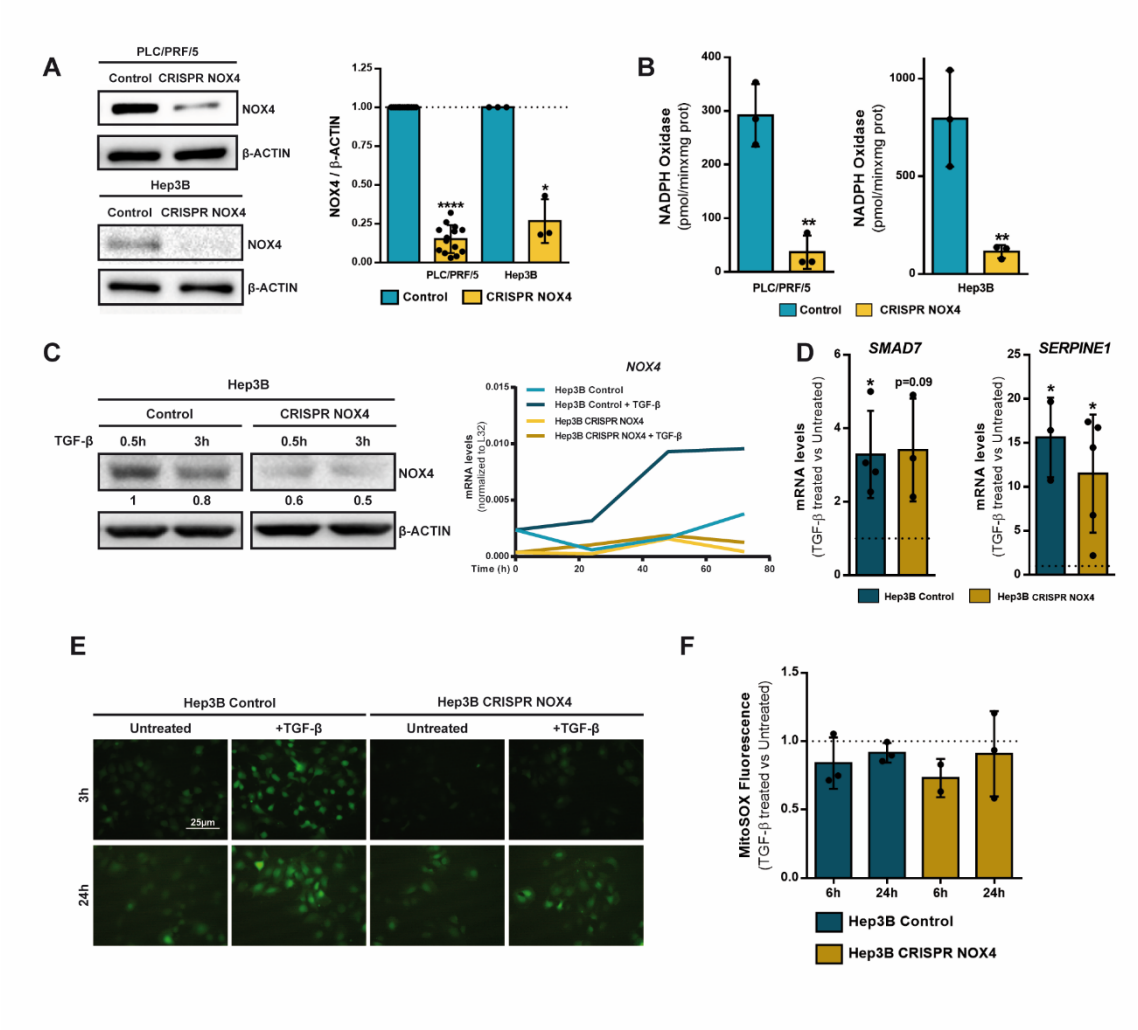
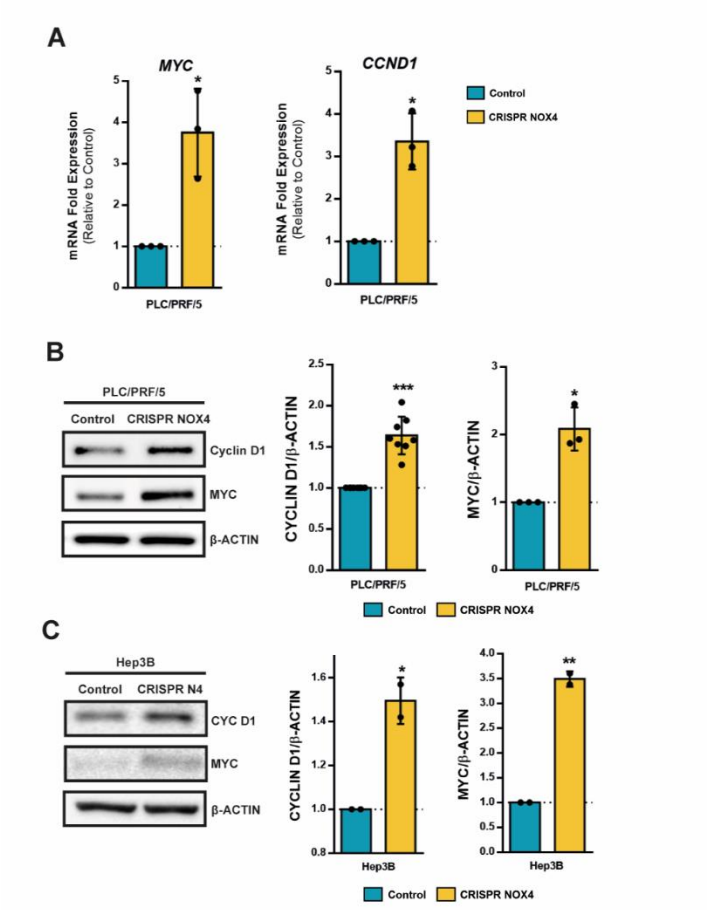


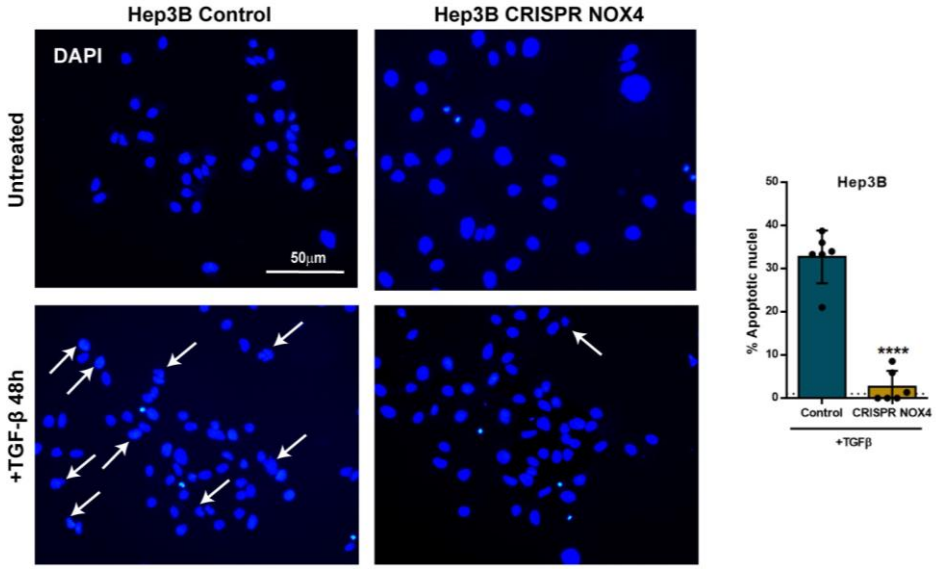
Supplementary Figure 1. Strategy to silence NOX4 with CRISPR/Cas9 technology in HCC cells. A) Design of RNA target guides (by bioinformatics tools) and cloning in the Cas9 vector (PX459) that contains resistance to puromycin. **B)** Selection of several clones after transformation with the different target guides: analysis of the insertion of the guide in the Cas9 vector by DNA sequencing (**top**) and restriction enzymes (**bottom**). **C)** Transfection of the HCC cell line with the different RNA target guides #1 (gRNA#1) or #2 (gRNA#2) and, after puromycin selection, T7 Endonuclease Assay to reveal if the Cas9 vector had worked correctly on the genomic DNA.



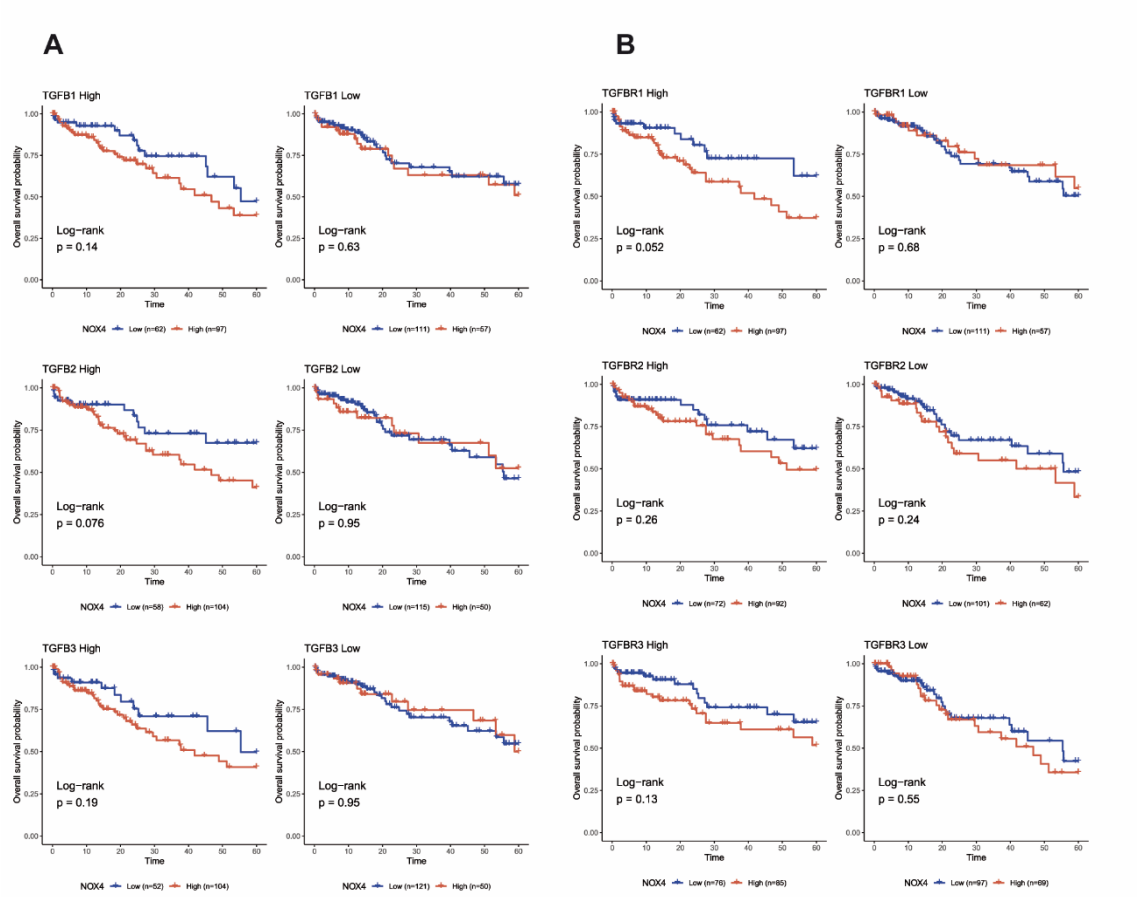
Supplementary Figure 2. Characterization of the cellular models used in this study. PLC/PRF/5 and Hep3B cells were stably transfected with either with a nonspecific vector (Control) or a mixture of both RNA guides #1 and #2 simultaneously (CRISPR NOX4) **A)** NOX4 protein levels analyzed by western blot. β-Actin was used as loading control. Representative experiment (**left**) and densitometric quantification of NOX4 levels relative to β-Actin (**right**). Data are Mean ± SD, n≥3). **B)** Analysis of NADPH oxidase activity, expressed as picomoles per minute per µg of protein. Data are Mean ± SD (n=3). **C)** NOX4 expression. Protein levels analyzed by western blot after TGF-β treatment at 0.5 and 3h. β-Actin was used as loading control (**left**). *NOX4* mRNA expression analysed by RT-qPCR, normalized to housekeeping gene *L32*, after TGF-β treatment at 24, 48 and 72 hours (**right**). Representative experiments are shown. **D)** *SMAD7* and *SERPINE1* mRNA expression analysed by RT-qPCR, normalized to housekeeping gene *L32*, after 48h TGF-β treatment, represented as fold induction (TGF-β-treated versus untreated cells). Data are mean ± SD (n=6). **E)** Analysis of intracellular ROS content by H_2DCFDA after TGF-β treatment. Scale bar, 25µm. **F)** Mitochondrial O_2^- analyzed fluorometrically using MitSOX™. Results are expressed as relative to each control. Data are mean ± SD (n≥3). Statistical analysis, where indicated: *p<0.05 **p<0.01, ***p<0.001, comparing CRISPR NOX4 cells versus CRISPR Control in A and B and TGF-β-treated versus untreated condition in D.



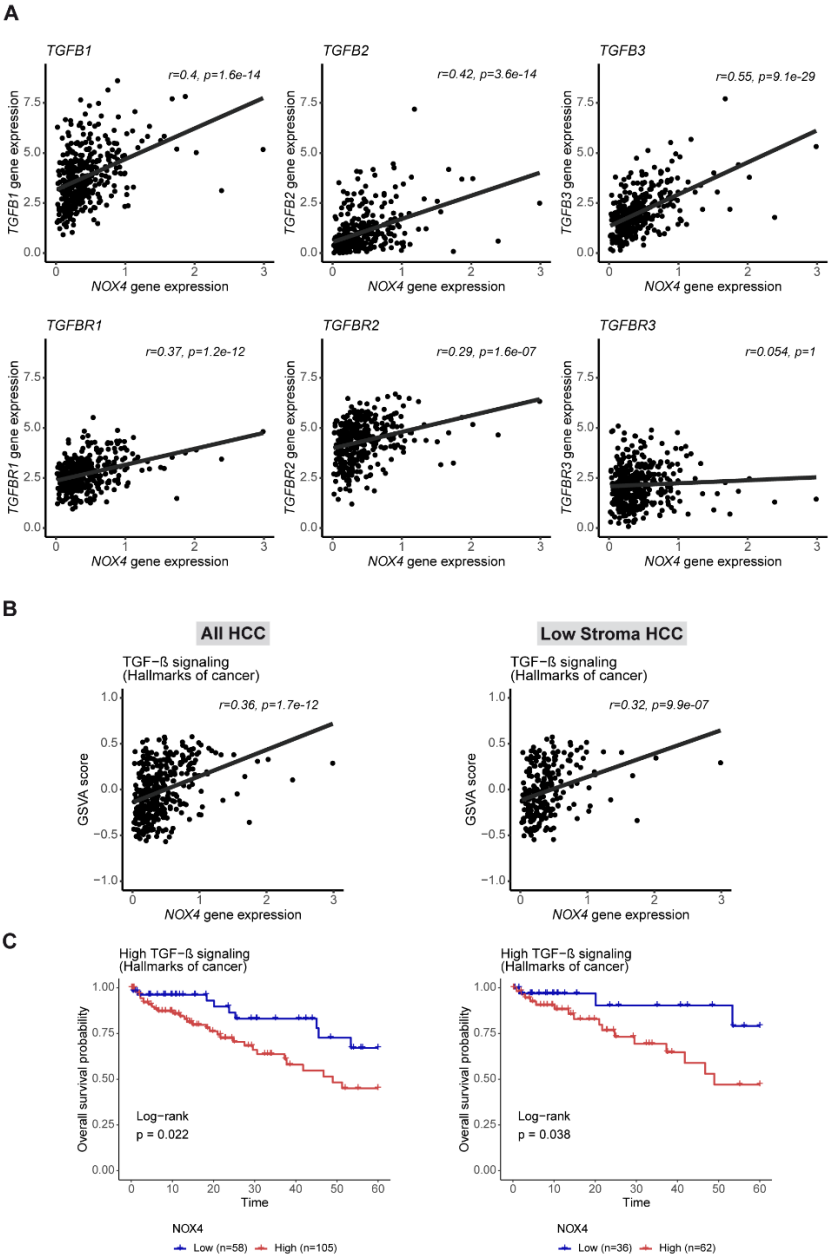
Supplementary Figure 3. *MYC* and C*CND1* (Cyclin D1) expression levels in HCC (Control and CRISPR NOX4) cells. A) *MYC* and *CCND1* mRNA expression analysed by RT-qPCR, normalized to housekeeping gene *L32*, in PLC/PRF/5 cells. **B-C)** C-MYC and Cyclin D1 protein levels analyzed by Western blot in both PLC/PRF/5 (B) and Hep3B (C) cells. β-Actin was used as loading control. Representative experiment (**left**) and densitometric quantification of protein levels expressed as relative to β-Actin (**right**). Data are mean ± SD (n≥3). *p<0.05 **p<0.01, ***p<0.001.



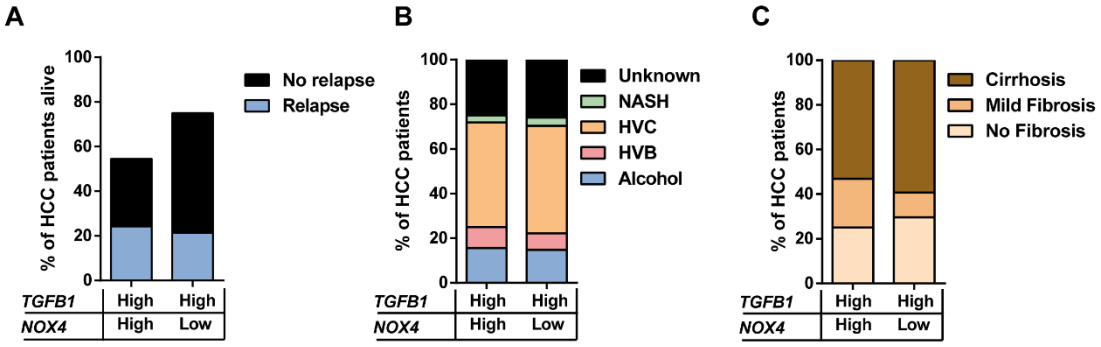
Supplementary Figure 4. Role of NOX4 on TGF-β-induced apoptosis. Analysis of fragmented nuclei (arrows) after DAPI (blue) staining in cells untreated or treated during 48 h with TGF-β. Representative images (**left**) and quantitative analysis (using ImageJ software), each dot representing one field (**right**).



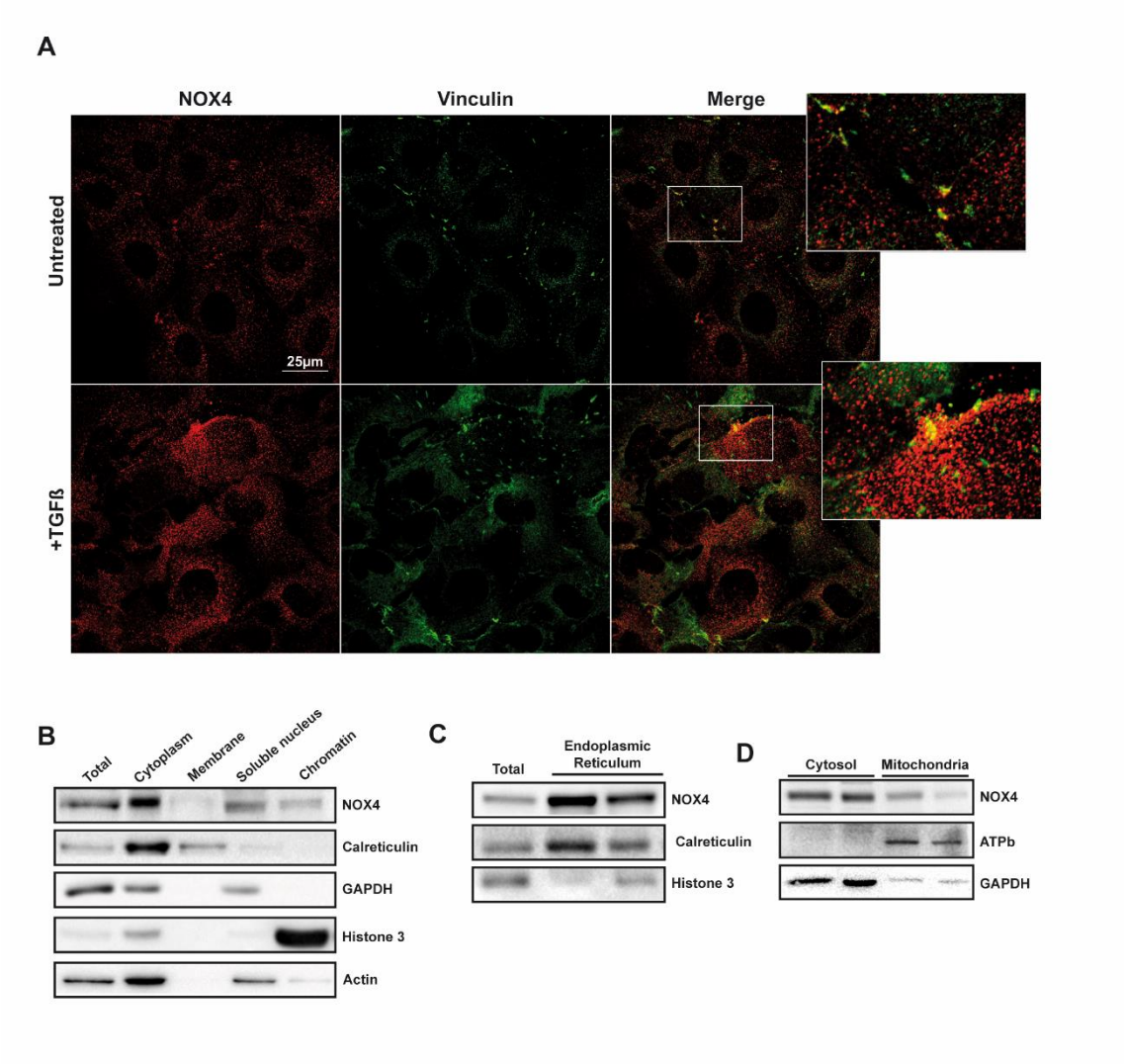
Supplementary Figure 5. Kaplan-Meier curve for overall survival (OS) probability for *NOX4*-low versus *NOX4*-high HCC patients, stratified by TGF-β ligands and receptors expression levels. Data from TCGA-LIHC public data base (n=327). **A)** TGF-β ligands**:** OS when *TGFB1*, *TGFB2* or *TGFB3* are high (**left**) or low (**right**). **B)** TGF-β receptors: OS when *TGFBR1*, *TGFBR2* or *TGFBR3* are high (**left**) or low (**right**). Genes are categorized using the median, and p-values are derived from a log-rank test.



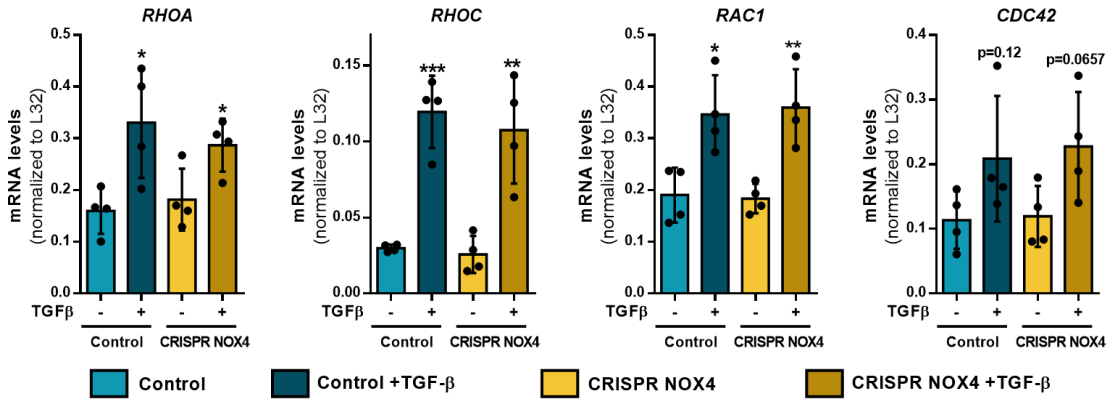
Supplementary Figure 6. In silico analysis of the correlation of NOX4 expression and genes encoding TGF-β ligands, receptors, and a TGF-β-signalling gene signature (Hallmarks of Cancer) (see Table 3)**.** Data from TCGA-LIHC public data base (n=327). **A)** Pearson correlation analysis between *NOX4* gene expression and *TGFB1*, *TGFB2* or *TGFB3* (**top**) or *TGFBR1*, *TGFBR2* or *TGFBR3* (**bottom**). **B)** Pearson correlation analysis between TGF-β signalling (quantified using Gene Set Variation Analysis (GSVA) score) and *NOX4* gene expression. Analysis done with all the HCC samples (**left**) or those with low stromal content (**right**). **C)** Kaplan-Meier curve for overall survival probability for *NOX4*-low versus *NOX4*-high patients when "TGF-β-signalling Hallmarks of Cancer" gene signature is high (above the median GSVA score). NOX4 is categorized using the median, and log-rank test is used to assess statistical differences. Analysis done with all the HCC samples (**left**) or those with low stromal content (**right**).



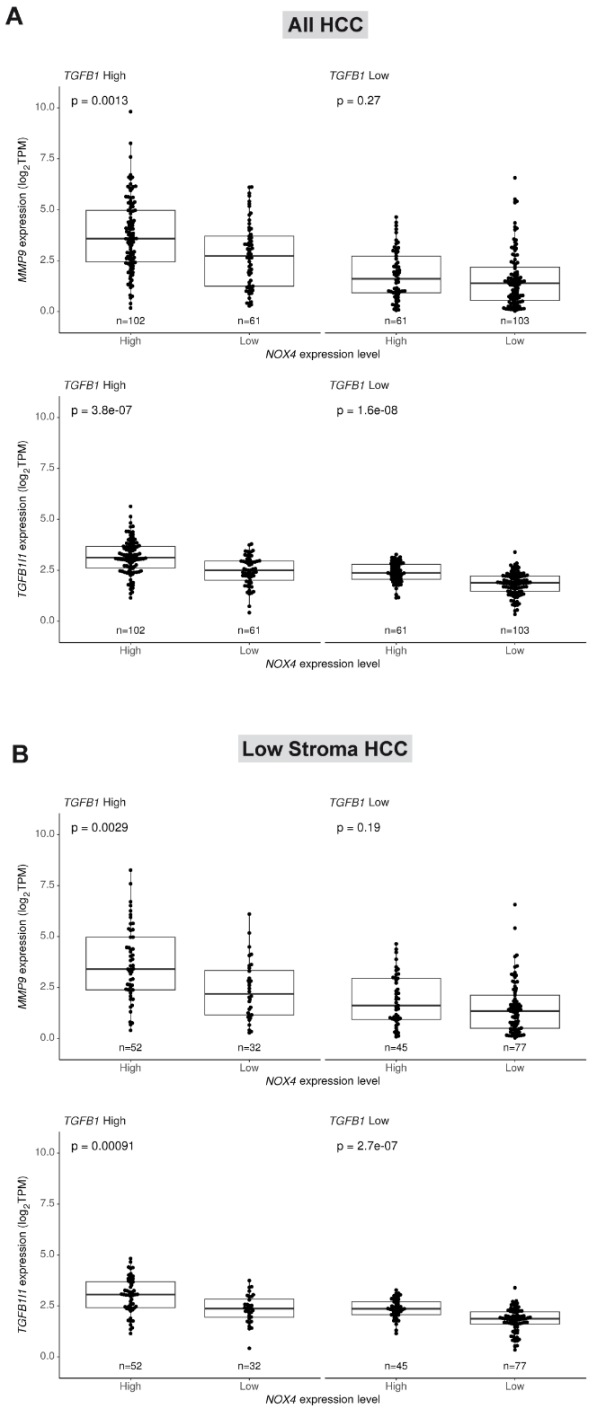
Supplementary Figure 7. Impact of the expression of NOX4 on relapse, fibrosis and aetiology in HCC patients with high expression of *TGFB1*. Analysis performed in alive patients at the time of analysis.0 **A)** Percentage of patients that suffered relapse versus those that did not. **B)** Percentage of HCC patients with different aetiologies: Alcohol, HVB, HVC, NASH or Unknown origin. **C)** Percentage of HCC patients presenting fibrosis, mild fibrosis or cirrhosis.



Supplementary Figure 8. NOX4 protein localization in different cellular compartments. Analysis made in Hep3B Control cells. **A)** Immunofluorescence of NOX4 (red) and Vinculin (green) in cells either untreated or treated during 48h with TGF-β. Representative images are shown. Scale bar, 25µm. **B)** NOX4 protein levels analyzed by western blot in different subcellular fractions of untreated PLC/PRF/5 cells, extracted as described in the Material and Methods section. **C**) Similar analysis in total or Endoplasmic Reticulum (ER) fractions and **D)** in Cytosol and Mitochondria fractions. Marker proteins to follow fractionation were: Calreticulin for endoplasmic reticulum (ER), GAPDH for cytosolic compartment, ATPb for mitochondria and Histone 3 for chromatin. Images representative or at least 3 independent experiments.



Supplementary Figure 9. NOX4 silencing in HCC Hep3B cells do not affect the response to 48h TGF-β treatment in terms of RhoGTPases family gene expression. *RHOA*, *RHOC*, *RAC1* and *CDC42* mRNA expression analysed by RT-qPCR, normalized to housekeeping gene *L32*, after TGF-β treatment at 48 hours. Data are Mean ± SD (n=4). *p<0.05 **p<0.01, ***p<0.001.



Supplementary Figure 10. In silico analysis of the expression of *MMP9* and *TGFB1I1*. **A)** Boxplot of *MMP9* (**top**) or *TGFB1I1* (**bottom**) gene expression for *NOX4*-low versus *NOX4*-high patients when *TGFB1* expression is low or high. Analysis done with all the HCC samples. **B)** Boxplot of *MMP9* (**top**) or *TGFB1I1* (**bottom**) gene expression for *NOX4*-low versus *NOX4*-high patients when *TGFB1* expression is low or high. Analysis done with low stromal content samples. Data from TCGA-LIHC public data base (n=327). P-values from a Mann-Whitney U test, adjusted for multiple testing.