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1 **MiR-30e-3p influences tumor phenotype through *MDM2/TP53* axis and predicts sorafenib**
2 **resistance in hepatocellular carcinoma**

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18

19 **Running title:** MiR-30e predicts sorafenib escape in HCC

20 **Key words:** HCC, microRNA, miR-30e, Sorafenib

21

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27

28 **Conflict of interest:** The Authors have no conflict of interest to declare

29

30 **Abstract**

31 The molecular background of hepatocellular carcinoma (HCC) is highly heterogeneous and
32 biomarkers predicting response to treatments are an unmet clinical need. We investigated miR-
33 30e-3p contribution to HCC phenotype and response to sorafenib, as well as the mutual
34 modulation of *TP53/MDM2* pathway, in HCC tissues and preclinical models. MiR-30e-3p was
35 downregulated in human and rat HCCs and its downregulation associated with *TP53* mutations.
36 *TP53* contributed to miR-30e-3p biogenesis and *MDM2* was identified among its target genes,
37 establishing a miR-30e-3p/*TP53/MDM2* feedforward loop and accounting for miR-30e-3p dual
38 role based on *TP53* status.

39 EpCAM, PTEN and p27 were demonstrated as miR-30e-3p additional targets mediating its
40 contribution to stemness and malignant features. In a preliminary cohort of HCC patients treated
41 with sorafenib, increased miR-30e-3p circulating levels predicted the development of resistance.

42 In conclusion, molecular background dictates miR-30e-3p dual behavior in HCC. Mdm2
43 targeting/p53 axis plays a predominant tumor-suppressor function in wild type *TP53* contexts,
44 whereas other targets such as PTEN, p27 and EpCAM gain relevance and mediate miR-30e-3p
45 oncogenic role in non-functional *TP53* backgrounds. Increased circulating levels of miR-30e-3p
46 predict the development of sorafenib resistance in a preliminary series of HCC patients and
47 deserve future investigations.

48

49 **Statement of significance**

50 The dual role of miR-30e-3p in hepatocellular carcinoma clarifies how the molecular context
51 dictates the tumor suppressor or oncogenic function played by microRNAs.

52

53 **Introduction**

54 Hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancers representing the
55 second leading cause of cancer mortality with increasing incidence in western countries [1].
56 HCC is characterized by a high inter- and intra-individual tumor heterogeneity: the former
57 ascribed to different risk factors and genomic contexts [2] and the latter to clonal evolution of
58 cancer cells [3], both contributing to limited targeted therapy efficacy. Primary resistance and
59 escape from antitumor strategies remain poorly understood and no biomarker predicting response
60 to sorafenib or other targeted treatments has been identified so far, highlighting the need for
61 novel tools to allocate patients to the best treatment.

62 The deregulation of tumor-specific microRNAs (miRNAs) with tumor suppressor or oncogenic
63 properties has been described [4, 5]. Our group and others demonstrated the key role of
64 deregulated miRNAs in tumor progression and metastasis [6, 7], as well as their involvement in
65 drug resistance phenotype [8-10]. Aberrant expression of miR-30 family members is a frequent
66 event in solid tumors [11, 12] displaying a prominent connection with other deregulated
67 miRNAs in neoplastic tissues [13]. The miR-30 family contains five members encoded by six
68 genes located on three distinct loci of the human genome (chromosomes 1, 6 and 8) giving rise
69 to six mature miRNAs (miR-30a, -30b, -30c-1, -30c-2, -30d, -30e) with the same seed sequence.
70 The downregulation of these miRNAs was associated with proliferation and invasion, as well as
71 with induction of epithelial-to-mesenchymal transition (EMT), exerted by direct targeting of
72 EMT-associated factors [14, 15] and cell adhesion molecules [16]. Remarkably, besides studies
73 supporting the tumor suppressor role of miR-30 family, other studies point to its opposite
74 behavior [17, 18].

75 *TP53* is one of the most frequently mutated gene in human cancers with about 30% of affected
76 HCC cases [19]. *TP53* regulates miRNA expression by acting at two different levels: on one
77 hand, it activates their transcriptional regulation [20-22] and, on the other hand, it interferes with
78 their maturation process by interacting with key molecules, such as Drosha and p68 RNA

79 helicase [23]. Notably, *TP53* regulates the expression of miR-30 family members contributing to
80 cell invasion and distal spreading as reported in colorectal and breast cancers [24, 25].
81 Despite the prominent role of miR-30 family as tumor suppressor miRNAs, there are conflicting
82 data regarding its aberrant expression in HCC. Previous studies reported the downregulation of
83 miR-30a and miR-30e and the upregulation of miR-30d [26-28]. Although miR-30 was reported
84 as participating in hepatobiliary development [29], its functional roles in HCC remain poorly
85 understood. Here, we investigated the expression of miR-30 family members in two HCC patient
86 cohorts, in a HCC animal model and in HCC cells, and focused on miR-30e-3p, characterizing
87 its biological activity as well as its involvement in drug resistance and modulation of
88 *MDM2/TP53* and *PTEN/AKT* axes. Moreover, we investigated circulating miR-30e-3p levels in a
89 preliminary series of HCC patients and in preclinical models in order to evaluate its contribution
90 as a non-invasive biomarker of treatment response.

91 **Materials and Methods**

92 **HCC study cohorts**

93 HCC and cirrhotic tissues were obtained from two independent cohorts of patients undergoing
94 liver surgery for HCC at the Department of Surgery and Transplant Unit of St. Orsola-Malpighi
95 University Hospital of Bologna. The discovery cohort consists of 22 patients, whereas the
96 validation cohort consists of 48 patients, all enrolled at the Department of Surgery of St. Orsola-
97 Malpighi University Hospital. We collected tissue samples at surgery and stored as previously
98 described [30]. Local ethics committee of St. Orsola-Malpighi University Hospital approved this
99 study (138/2015/O/Tess). Normal liver tissues were from patients undergoing liver surgery for
100 traumatic lesions or haemangioma resection. The clinical characteristics of patients are detailed
101 in Supplementary Table S1. *TP53* mutations were identified as previously described [20].

102 A further cohort of sorafenib-treated advanced HCC patients (Table S2) was tested for serum
103 miR-30e-3p levels before treatment and at two-month follow-up assessment. Sera samples,
104 obtained after the local committee approval (271/2012/O/Oss), were processed as previously
105 described [31]. Informed written consent has been obtained from patients enrolled in this study.

106

107 **HCC animal models**

108 The diethyl-nitrosamine (DEN)-induced HCC rat model and the xenograft model were
109 established and treated as previously described [9]. Total RNA was extracted from frozen tissues
110 by using TRIzol Reagent (Invitrogen) and was analyzed by microarray and QPCR. Local ethics
111 committee approved the study protocols (14/70/12 and 23/79/14).

112

113 **Microarray analysis**

114 RNAs from 22 rat samples (12 HCCs, 8 surrounding livers and 2 normal livers) were hybridized
115 on Agilent rat whole-genome miRNAs microarray (#G4471A_046066 Release 19.0, Agilent

116 Technologies). One-color gene expression was performed according to the manufacturer's
117 procedure. Raw data are available in ArrayExpress repository (accession number E-MTAB-
118 7624).

119 Technical details are described in Supplementary section.

120

121 **Cell culture and treatments**

122 HCC cell lines were cultured as previously described [9]. Starvation was obtained culturing cells
123 in media without FBS for 48-60 hours. Cells were treated with 5.0-7.5 μ M sorafenib-tosylate
124 (Bayer) or Nutlin-3 (Sigma-Aldrich) or with 1.0-2.0 μ g/ml of doxorubicin (Pfizer) for 48 hours.
125 Cell transfection and proliferation, clonogenic and sphere formation assays are detailed in
126 Supplementary Material. Genetics Unit at S.Orsola-Malpighi Hospital performed HCC
127 authentication and cell identification was obtained by online STR analysis
128 (<https://www.dsmz.de/>).

129

130 **Reporter assays**

131 3' untranslated regions (3'UTR) of *MDM2*, *CDKN1B/p27*, *PTEN* and *EpCAM* mRNAs were
132 amplified by PCR as reported in Supplementary Table S2. Mutagenesis of miR-30e-3p seed
133 sequence was performed by using QuikChange II Site-Directed Mutagenesis Kit (Agilent
134 Technologies) following manufacturer's instruction (Supplementary Table S3). Sanger
135 sequencing verified mutated sequences. Dual-luciferase reporter and p53 activity assays were
136 performed as previously described [32].

137

138 **Real-time PCR**

139 TaqMan MicroRNA Assays (Applied Biosystems) were used to evaluate miRNA expression, as
140 previously described [30]. RNU6B was used as housekeeping gene. Quantitative PCR (qPCR)

141 was used for gene expression analysis. β -actin and GAPDH housekeeping genes were considered
142 for gene normalization. QPCR experiments were run in triplicate. Primers and conditions are
143 detailed in Supplementary Table S4.

144

145 **Western blot**

146 Western blot (WB) was used to analyze protein extracts (30 μ g) from cell and tissues with
147 antibodies reported in Supplementary Table S5. ChemiDocTM XRS+ (Image LabTM Software,
148 Bio-Rad) was employed to quantify digital images of X-ray films. WB analysis was performed
149 in duplicate.

150

151 **Chromatin immunoprecipitation and electrophoretic mobility shift assay**

152 HepG2 cells were subjected to chromatin immunoprecipitation (ChIP) with a polyclonal p53
153 antibody (Novocastra) as previously described [21]. Primers and conditions are reported in
154 Supplementary Table S4. Amplicon design has been detailed in Supplementary Material.
155 Electrophoretic mobility shift assay (EMSA) was performed with nuclear extract (NE) from
156 Nutlin-3 treated HepG2 cells by using LightShift Chemiluminescent EMSA Kit (Thermo
157 Scientific) as previously described [21]. Probe sequences are reported in Supplementary Table
158 S6.

159

160 **Flow cytometry**

161 Annexin-V assay was performed in duplicate by flow cytometry (FACSaria I, BD) as previously
162 reported [33]. Annexin-V assay in miR-30e-3p overexpressing HepG2 cells was assessed on
163 Cytoflex S (Beckam Coulter). Immunophenotype analysis of EpCAM was performed by using
164 CD326 monoclonal antibody (MH99)-Alexa Fluor 488 (eBioscience).

165

166 **Cell invasion and wound healing assay**

167 Real-time cell invasion, performed on xCELLigence DP instrument (ACEA), and wound healing
168 assays were executed as previously described [34].

169

170 **Caspase activity assay**

171 Caspase pathway activation was evaluated by Caspase-Glo 3/7 assay (Promega) according to
172 manufacturer's instructions. Each sample was performed in quadruplicate in two independent
173 experiments.

174

175 **Serum and exosome miRNA extraction**

176 Isolation of circulating miRNAs from exosomes, cell culture supernatant, and serum was
177 executed as previously reported [9, 31].

178

179 **Statistical analysis**

180 Differences between two or more groups were analyzed using unpaired Student's t-test or
181 ANOVA. Tukey's post hoc test was used for comparisons among groups after ANOVA analysis.
182 Pearson's correlation coefficient was used to investigate relationships between two variables.
183 Time to recurrence (TTR) curve based on miR-30e-3p levels was computed by Kaplan-Meier
184 product-limit method and compared using a log-rank test. Paired t-test was used to evaluate the
185 relationship between circulating miR-30e-3p and response to sorafenib in HCC patients analyzed
186 both before and on treatment. Reported p-values were two-sided and considered significant when
187 lower than 0.05. Statistical calculations were executed using SPSS version 20.0 (SPSS inc). *
188 $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

189 **Results**

190 **MiR-30e-3p is downregulated in HCC and associates with *TP53* status**

191 We previously reported a deregulation of miR-30 family members in histologically aggressive
192 HCCs [35]. Here, we confirmed in a first patient cohort, a decrease of miR-30e-3p in 73% of
193 HCCs in comparison to matched cirrhotic livers (t-test; $p=0.0002$), showing a mean
194 downregulation of 1.7-fold (**Figure 1A**). Similarly, a decrease of miR-30e-5p and miR-30a-3p
195 was detected in 64% of tumors with an average 1.6-fold change (t-test; $p=0.003$ and $p=0.008$,
196 respectively) (**Figure 1B, C**). A positive correlation between these miR-30 family members was
197 detected in HCC and cirrhotic human samples (**Figure S1A-F**), suggesting common regulation
198 mechanisms for these miR-30 family members.

199 We next tested the DEN-induced HCC rat model [9] by microarray analysis on 12 HCCs and 10
200 non-cancerous livers. The downregulation of five miRNAs belonging to miR-30 family was
201 observed in tumors compared to surrounding livers (**Figure S2 and Table S7**). In rat HCC, miR-
202 30e-3p decreased in 64% of tumors, showing a 1.5-fold change (t-test; $p=0.034$), mirroring
203 findings from human HCCs (**Figure 1D**). MiR-30e-5p and miR-30a-3p were downregulated in
204 59% and 32% of rat HCCs, showing no difference between tumor and non-tumor samples
205 (**Figure 1E, F**). The discrepancy between human and rat HCCs might be ascribed to the lack of
206 cirrhotic microenvironment in the rat model.

207 In the validation cohort, we confirmed miR-30e-3p, miR-30e-5p and miR-30a-3p
208 downregulation in HCCs with respect to both matched cirrhosis (Tukey's post hoc test; $p=0.012$,
209 $p=0.013$ and $p=0.025$, respectively) and normal livers (Tukey's post hoc test; $p=0.046$, $p=0.001$
210 and $p=0.044$, respectively) (**Figure 1G-I**). Here, we focused on miR-30e-3p, which is the most
211 downregulated miRNA in human and rat HCCs.

212 Since *TP53* is mutated in about 30% of HCCs and it establishes complex regulatory networks
213 with miRNAs [1, 36], we investigated the relationship between *TP53* status and miR-30e-3p in

HCC. Lower miR-30e-3p levels were observed in *TP53* mutated HCCs while no difference was found for primary miR-30e (pri-miR-30e) levels (**Figure 1J, K**). Remarkably, a negative correlation between primary and mature miR-30e levels was found in HCCs (Pearson's correlation; $R=-0.32$) (**Figure S3A**), suggesting a role for post-transcriptional mechanisms in miR-30e biogenesis regulation. Interestingly, after splitting HCCs according to *TP53* status, a negative correlation was confirmed between primary and mature miRNA levels in *TP53* mutated cases, while a trend towards a positive correlation was found in *TP53* WT cases (**Figure S3B, C**). These findings suggest an impairment of miR-30e-3p maturation by mutant p53 isoforms which were previously reported to interfere with the functional assembly of Drosha/p68 processing complex [23].

***TP53* influences miR-30e-3p transcription and biogenesis in HCC cells**

To start dissecting p53 involvement in miR-30e biogenesis in HCC, we modulated its expression in *TP53* WT HepG2 cells by using different strategies. First, we investigated the influence of p53 silencing on miR-30e primary transcript and mature isoforms. As showed in Figure 2A, a decrease of primary and mature miR-30e-3p and miR-30e-5p levels was detected in p53-silenced cells. MiR-34a was used as a positive control since it represents the first identified p53-target miRNA [37], whereas p53 silencing was verified by WB and qPCR analyses of target genes (**Figure 2A**). Secondly, we investigated the effect of p53 overexpression in HepG2 cells following transfection of a p53-overexpressing vector or treatment with Nutlin-3, a *MDM2* inhibitor. In line, an increase of miR-30e primary transcript and mature miRNA isoforms was detected in both experimental settings (**Figure 2B, C**). Notably, Nutlin-3 administration led to a stronger p53 transcriptional activation with respect to p53-overexpressing vector, as confirmed by the extent of variation of *CDKN1A/p21* and *MDM2* mRNAs. In turn, this resulted in higher pri-miR-30e levels, but not mature isoforms (**Figure 2B, C**), letting us to speculate that

mdm2/p53 axis might have an additional role in miR-30e biogenesis. To investigate p53 involvement in miRNA maturation, we quantified primary and mature miR-30e levels in p53-deleted (CRISPR/Cas9 technology) HepG2 cells and p53-null Hep3B cells following Nutlin-3 treatment. In line with p53 absence, no increase of miR-30e primary transcript was observed, whereas increased mature miRNA isoforms were detected in both treated cell lines (**Figure 2D, E**), confirming p53 role in miRNA processing impairment during Nutlin-3 administration. Since Dicer1 promoter contains several p63/p53 responsive elements [38], we investigated Dicer1 expression in HepG2 cells following p53 overexpression or Nutlin-3 treatment in both p53 WT and p53-deleted cells. A downregulation of Dicer1 was observed in Nutlin-3 treated HepG2 cells, but not in p53-overexpressing cells, which is in line with miRNA maturation impairment detected after Nutlin-3 administration in p53 WT cells (**Figure S3D, E**). On the contrary, higher Dicer1 levels were detected in p53-deleted HepG2 and p53 null Hep3B cells (**Figure S3F, G**), suggesting that mdm2 inhibition by Nutlin-3 influences miR-30e processing by regulating Dicer1 transcription in a p53-dependent and independent manner.

To go deeper into p53-mediated mechanisms regulating miR-30e expression, we overexpressed WT and mutant (truncated, dominant negative) *TP53* isoforms in p53-deleted Hep3B and p53 WT HepG2 cells, respectively. As displayed in Figure 2F, an increase of primary and mature miR-30e isoforms was observed in *TP53*-overexpressing Hep3B cells confirming its role in miRNA transcription. Interestingly, mutant p53 expression in HepG2 cells decreased miR-30e transcription and, consequently, mature isoforms (**Figure 2G**), suggesting a negative role for p53 mutations in miR-30e regulation. These findings demonstrate that *TP53* WT isoform is necessary to induce miR-30e transcription in HCC cells and that p53/mdm2 axis is involved in a multi-step regulation of miR-30e biogenesis.

To prove p53 binding to hypothetical consensus elements in proximity of miR-30e precursor region, a ChIP experiment was conducted in control and Nutlin-3 treated HepG2 cells. Six amplicons containing *TP53* hypothetical binding sites were designed in a DNA region spanning

from -4000 to +1000 nucleotides, considering +1 the first nucleotide of miR-30e precursor (Figure S3H). This region was chosen based on described promoter distance for intronic miRNA [39] and due to the presence of histone modifications (Figure S3I). The ChIP analysis showed an enrichment of three out of six p53 hypothetical binding sites, namely BS1, BS2 and BS6, in Nutlin-3 treated cells only (Figure S3J), demonstrating that p53 activation strengthen its binding to miR-30e-3p genomic region favoring pri-miR-30e regulation. EMSA assay further confirmed p53 binding to hypothetical consensus sites identified in the ChIP experiment, showing a specific shift for BS1, BS2 and BS6 probes in presence of nuclear extracts from Nutlin-3 treated HepG2 cells (Figure S3K).

Notably, a positive correlation (Pearson's correlation; $R=0.71$) exists between pri-miR-30e and its host gene, nuclear transcription factor Y subunit gamma (*NFYC*, NM_014223) in HCCs, whereas a negative correlation (Pearson's correlation; $R=-0.76$) was found between mature miR-30e-3p and *NFYC*, outlining the relevance of maturation process in the regulation of miR-30e levels (Figure S3L, M).

279

280 **MiR-30e-3p establishes a *TP53* positive feedback loop through *MDM2* targeting**

To investigate the interplay between miR-30e-3p and *TP53*, we interrogated bioinformatics tools and identified the principal p53 negative regulator, *MDM2* (NM_002392), as a hypothetic miR-30e-3p target gene containing three complementary binding sites (Figure 3A). We quantified miR-30e-3p levels in seven HCC cell lines and compared its expression with mean values observed in HCCs, cirrhosis and normal livers (Figure 3B). In order to avoid a confounding effect due *TP53/MDM2* auto-regulatory loop, we performed a functional analysis in HCC cell lines with mutated or null p53 isoform. Huh-7, Hep3B and SNU475 cells were chosen based on their low/intermediate miR-30e-3p basal levels, whereas SNU449 cells were selected because of their high basal levels (Figure 3B). MiR-30e-3p overexpression in Huh-7, Hep3B and SNU475

cells decreased mdm2 mRNA and protein levels, whereas miRNA silencing in SNU449 cells increased its mRNA and protein expression (**Figure 3C-F**). Subsequently, we explored *MDM2* targeting by miR-30e-3p in HepG2 cells. We observed increased mRNA and protein levels following transient miRNA silencing in p53 WT and silenced (1.3 and 1.5-fold, respectively) HepG2 cells (**Figure S4A, B**). To assess if miR-30e-3p regulation of *MDM2* is long lasting, we evaluated its expression in miR-30e-3p stably silenced (MZIP-30e-3p) HepG2 cells and detected increased protein levels (**Figure S4C**), highlighting a prolonged post-transcriptional regulation. Finally, we verified miRNA/mRNA interaction by performing a reporter assay in HepG2 cells. MiR-30e-3p co-transfection decreased luciferase activity of WT *MDM2*-3'UTR vectors, but not that of mutant ones, proving miRNA direct interaction with its complementary sequence in *MDM2* mRNA (**Figure 3G**). To verify the existence of a possible miR-30e/*MDM2*/*TP53* feedback loop, we measured p53 transcriptional activity and expression in WT and mutant *TP53*-bearing cells following miR-30e-3p overexpression. An increase of luciferase signal and p53 protein expression was registered in miR-30e-3p transfected HepG2 cells; whereas no change was observed in Huh-7 cells harboring an inactive p53 isoform (**Figure 3H, S4D**). These data demonstrated that, in p53 WT contexts, miR-30e-3p increases p53 expression and activity by targeting mdm2, leading to a feedforward loop sustained by p53-dependent transcription of miR-30e-3p itself. Conversely, in *TP53* mutated backgrounds this positive loop is hampered, suggesting that other targets might mediate miR-30e-3p functions in these contexts.

309

310 **MiR-30e-3p influences cell proliferation and invasion of HCC cells by targeting *MDM2*,**

311 ***PTEN* and *CDKN1B/p27***

The regulation of cell proliferation and invasion by miR-30 family members has been reported in tumors [40, 41]; nevertheless, miR-30e-3p, the most downregulated miR-30 member in our patient cohorts and preclinical model, was not previously studied. Besides *MDM2*, a

315 bioinformatics analysis highlighted *p27* and *PTEN* among miR-30e-3p hypothetic targets
 316 (**Figure S5A**). Due to their key role in hepatocarcinogenesis, it is conceivable that miR-30e-3p
 317 establishes multi-target networks driving HCC phenotype. QPCR and WB analyses in
 318 transfected HCC cells proved *p27* and *PTEN* inhibition by miR-30e-3p at mRNA and protein
 319 levels (**Figure S5B**). A reporter assay showed decreased luciferase activity of p27 and PTEN
 320 3'UTR WT vectors upon miR-30e-3p overexpression, demonstrating that miR-30e-3p directly
 321 regulates these targets through mRNA degradation (**Figure S5C**).

322 We subsequently explored the effect of miR-30e-3p on HCC cell proliferation by dissecting the
 323 contribution of mdm2/p53 axis and PTEN/AKT pathway. In order to investigate the former, we
 324 performed the proliferation assay in *TP53* WT and p53-silenced HepG2 cells. Cell growth
 325 inhibition (1.2-fold) occurred upon miR-30e-3p enforced expression in *TP53* WT cells, whereas
 326 an increase of cell proliferation (1.4-fold) was detected in *TP53*-silenced HepG2 cells (**Figure**
 327 **4A, B**), suggesting p53 as a pivotal factor modulating miR-30e-3p effect. The relevance of *PTEN*
 328 targeting on cell proliferation was next assessed in p53-silenced cells by evaluating *AKT*
 329 phosphorylation upon miR-30e-3p enforced expression. An increase of phospho-AKT occurred
 330 in both *TP53* WT and p53-silenced HepG2 cells highlighting AKT, beside p53, as pivotal factors
 331 mediating miR-30e-3p effects. We next assayed p53-mutated Huh-7 and p53-deleted Hep3B
 332 cells displaying high constitutive pten levels [20]. MiR-30e-3p overexpression increased
 333 proliferation of Huh-7 and Hep3B cells (1.9 and 2.1-fold, respectively). Enhanced AKT
 334 phosphorylation confirmed PTEN/AKT targeting as a trigger for proliferation in these non-
 335 functional p53 backgrounds (**Figure 4C, D**). To check whether p27 contributes to cell
 336 proliferation, we chose p53-mutated SNU475 cells displaying null pten levels [20]. MiR-30e-3p
 337 increased proliferation of SNU475 cells without changing phospho-AKT levels, but strongly
 338 decreasing p27 (**Figure 4E**). These findings support the crucial role of mdm2/p53, p27 and AKT
 339 in mediating miR-30e-3p regulation of cell proliferation. We hence analyzed cell cycle
 340 regulators p21 and p27 in miR-30e-3p overexpressing HCC cells. P21 modulation reflected p53

status, showing an increase in *TP53* WT HepG2 cells only, while it did not change in *TP53*-mutated cell lines (**Figure 4A-E**). Conversely, p27 downregulation was observed in all cell lines, confirming its targeting by miR-30e-3p (**Figure 4A-E**). Opposite proliferative changes induced by miR-30e-3p in HCC cell lines can be explained by its multi-target activity. Indeed, the inhibition of mdm2 prevents cell cycle acceleration through p53/p21 signaling in *TP53* WT contexts only. On the contrary, p27 and PTEN inhibition prevails in *TP53*-mutated/deleted backgrounds, leading to increased proliferation.

We next explored miR-30e-3p ability to modulate invasive properties of HepG2 and SNU449 cells. These cell lines were chosen due to high miR-30e-3p constitutive levels that allowed the investigation of miR-30e-3p stable silencing (**Figure S5D**) in a p53 WT (HepG2) and p53-mutated context (SNU449). No change was observed in miR-30e-3p silenced HepG2 cells, whereas decreased invasive and migratory potentials were detected in miRNA-silenced SNU449 cells (Student's t-test; $p < 0.0001$) (**Figure 4F, S5E**). Despite decreased phospho-AKT levels in both cell lines, their different invasion capability highlights cell context as pivotal in driving miR-30e-3p influence on cell aggressiveness.

356

357 **MiR-30e-3p influences stem cell properties of HCC cells through *EpCAM* targeting**

We investigated miR-30e-3p driven changes on stemness in HCC and observed a negative correlation between miR-30e-3p and both AFP and EpCAM mRNAs in HCC patients (Pearson's correlation; $R = -0.45$ and -0.36 , respectively) (**Figure 5A**) and cell lines (Pearson's correlation; $R = -0.62$) (**Figure S6A**). Analyzing HCCs according to *TP53* status, an even stronger correlation was observed in *TP53* mutated patients (Pearson's correlation; $R = -0.78$) (**Figure S6B**), suggesting a stronger activity of miR-30e-3p in the absence of miR-30e-3p/mdm2/p53 regulatory loop. An opposite behavior was detected for pri-miR-30e that positively correlated with EpCAM mRNA (Pearson's correlation; $R = 0.39$) (**Figure S6C**). Accordingly, a change of EpCAM mRNA

366 and immunophenotype was observed following miR-30e-3p modulation in HCC cells (**Figure**
 367 **5B and S6D**). To investigate how miR-30e-3p influences stem cell properties, we interrogated
 368 bioinformatics algorithms and identified two hypothetical miRNA binding sites in EpCAM
 369 3'UTR (**Figure S6E**). A decreased luciferase activity was observed for WT EpCAM-3'UTR
 370 vector in presence of miR-30e-3p overexpression (Student's t-test; $p=0.00045$), demonstrating a
 371 direct regulation of this target in HCC (**Figure 5C**). We next performed clonogenic and sphere
 372 formation assays in miR-30e-3p stably silenced (MZIP-30e-3p) p53 WT HepG2 and p53-
 373 mutated SNU449 cells (**Figure S5E, S6F**), chosen on the basis of their different stemness
 374 features (**Figure S6G**). The clonogenic assay displayed an increase of colony number (t-test;
 375 $p=0.0005$) and OD 595 (t-test; $p=0.001$) in MZIP-30e-3p HepG2 cells, whereas an opposite
 376 behavior was observed in SNU449 cells displaying a decreased colony number (t-test; $p<0.0001$)
 377 and OD 595 (t-test; $p=0.025$) in miR-30e-3p silenced cells (**Figure 5D, E**). Finally, we
 378 performed colony-forming unit and sphere formation assays in miR-30e-3p stably
 379 overexpressing Huh-7 cells exhibiting high stem cell characteristics and a mutated p53 isoform
 380 (**Figure S6F, G**). A decrease of colony and sphere number (t-test; $p=0.0033$ and $p=0.010$,
 381 respectively) was observed in miR-30e-3p-Huh-7 cells (**Figure 5F, G**). Similarly, miR-30e-3p
 382 silencing determined an increased sphere formation (Student's t-test; $p<0.0001$) in HepG2 cells
 383 (**Figure 5G, S6H**). Due to low expression of stem cell characteristics, SNU449 cells displayed
 384 no capability to sphere formation (**Figure S6I**). An increase of AFP and EpCAM levels was
 385 observed in MZIP-30e-3p HepG2-derived spheres, whereas their decrease was detected in
 386 pMXs-miR-30e-3p Huh-7-derived spheres (**Figure 5H**), confirming findings observed in HCC
 387 specimens and cell lines.

388 In primary HCCs, low miR-30e-3p expression associated with microvascular invasion (Student's
 389 t-test; $p=0.012$) and increased recurrence rate (log-rank test; $p=0.027$) (**Figure 5I, J**). These
 390 findings demonstrated that miR-30e-3p downregulation participates to boost stemness properties
 391 in HCC cells characterized by a stem-like phenotype.

392

393

394 **MiR-30e-3p regulates treatment response in HCC preclinical models through *MDM2* and**
395 ***PTEN* targeting**

396 Due to the relevance of miR-30e-3p targets in stressful conditions and drug response, we
397 evaluated apoptotic cell death in HepG2 and Huh-7 cells following starvation and doxorubicin or
398 sorafenib treatment. Firstly, we considered *TP53* WT HepG2 cells and observed that miR-30e-3p
399 overexpression had no effect in basal conditions (**Figure 6A**). On the contrary, it increased early
400 apoptosis (2.3-fold) following serum deprivation and both early and late apoptosis (1.3 and 7.8-
401 fold, respectively) in doxorubicin treated cells (**Figure 6B, C**), which is consistent with p53
402 activation in these conditions (**Figure S7A**). WB and caspase assay confirmed increased p53
403 expression, apoptotic markers and caspase-3/7 activity upon miR-30e-3p enforced expression in
404 serum-deprived and doxorubicin treated HepG2 cells (**Figure 6C-E**).

405 Similarly, sorafenib increased early apoptosis (3.1-fold increase) in miR-30e-3p transfected
406 HepG2 cells (**Figure 6F-H**). Since both sorafenib (**Figure S7A**) and miR-30e-3p induces p53
407 expression (**Figure 6G**), we wondered if p53 might account for increased apoptotic cell death in
408 miR-30e-3p overexpressing cells. To prove a p53-dependent response, we performed the same
409 assays in p53-silenced HepG2 cells. In this setting, miR-30e-3p overexpression decreased (4.2-
410 fold) early apoptotic cells (**Figure 6I-K**), demonstrating the central role of p53 in triggering
411 miR-30e-3p-mediated apoptosis. In agreement, miR-30e-3p overexpression in *TP53* deleted
412 (CRISPR/Cas9 technology) HepG2 cells determined a resistance to both starvation and sorafenib
413 (**Figure S7B-E**), confirming p53 as a determinant of miR-30e-3p duality in HCC. To
414 demonstrate the relevance of *PTEN/AKT* axis in the absence of p53, we coupled miR-30e-3p
415 inhibition with *PTEN* silencing in p53-deleted HepG2 cells. Annexin-V assay after sorafenib
416 administration showed that *PTEN* inhibition decreases early apoptosis (1.5-fold) in miR-30e-3p

417 silenced/p53-knockout HepG2 cells confirming PTEN/AKT pathway as a driver of sorafenib-
418 resistance in the absence of p53 (**Figure S7F, G**). Finally, since sorafenib triggers p27 in HepG2
419 cells (**Figure S7H**), we wondered whether p27 targeting by miR-30e-3p might influence
420 sorafenib response. Strikingly, when both *TP53* and *PTEN* were inhibited, miR-30e-3p
421 overexpression decreased early apoptotic cell population (1.4-fold) in sorafenib treated HepG2
422 cells, suggesting p27 participation to miR-30e-3p-mediated sorafenib resistance in p53-depleted
423 cells (**Figure S7I**). These findings confirm the importance of PTEN and p27 in driving miR-30e-
424 3p-associated phenotype in *TP53* mutated backgrounds. In line, miR-30e-3p overexpression in
425 *TP53* mutated Huh-7 cells determined a resistance to starvation, doxorubicin and sorafenib
426 administration (**Figure S8A-H**) and high miR-30e-3p levels associated with increased tumor cell
427 proliferation in sorafenib-treated Huh-7-derived xenograft mice (**Figure S8I, J**), confirming its
428 oncogenic role in p53-mutated cells.

429 In sorafenib-treated DEN-HCC rats, a downregulation of miR-30e-3p expression was found in
430 73% of nodules (Student's t-test; $p=0.02$) (**Figure 7A**). Sorafenib resistant tumors showed lower
431 miR-30e-3p tissue levels (Student's t-test; $p=0.004$) (**Figure 7B**), suggesting miR-30e-3p
432 downregulation as an adverse event with respect to sorafenib efficacy in this model. In
433 agreement, an inverse correlation between miR-30e-3p and tumor size (Pearson's correlation;
434 $R=-0.69$) (**Figure 7C**) and a positive correlation between miR-30e-3p and apoptotic markers
435 (Pearson's correlation; $R=0.70, 0.71$ and 0.49) were identified in treated rat HCCs (**Figure S9A-**
436 **C**). Finally, the positive correlation between miR-30e-3p and p21 levels in tumor tissues
437 (Pearson's correlation; $R=0.52$) (**Figure S9D**) from *TP53* WT rat DEN-HCCs [42], led us to
438 speculate that miR-30e-3p/mdm2/p53 axis might take part to sorafenib sensitization also in rat
439 tumors.

440

441 **Circulating miR-30e-3p levels predict treatment response in HCC**

442 Circulating miRNAs were proposed as biomarkers of treatment response [43] and TKIs, such as
443 gefitinib, promote exosomal secretion [44]. Thus, we assayed if sorafenib could modulate miR-
444 30e-3p extracellular levels. An exosome-mediated miR-30e-3p extrusion was confirmed in all
445 HCC cell lines treated by sorafenib, except for SNU449 cells. Sorafenib decreased intracellular
446 miR-30e-3p levels in sensitive cell lines (HepG2, Hep3B and Huh-7), while it increased miRNA
447 levels in the resistant (SNU475) one (**Figure 7D-F**). Aiming at identifying molecular
448 mechanisms underneath miR-30e-3p extrusion triggered by sorafenib, we focused on p53, a
449 known promoter of exosomal secretion [45]. We assayed p53 activity following sorafenib
450 treatment in WT *TP53* HepG2 cells, in SNU475 cells that harbor a double *TP53* heterozygous
451 mutation but maintain a residual *TP53* transcriptional activity and in SNU449 cells with a
452 mutated and transcriptionally inactive *TP53* isoform (**Figure 3B**). Sorafenib increased *TP53*
453 activity in both HepG2 and SNU475 cells, but not in SNU449 cells (**Figure 7G**). We next
454 performed *TP53* silencing and deletion in HepG2 cells and observed that p53 absence prevented
455 miR-30e-3p extracellular and exosomal rising (**Figure 7H and S9E**), confirming that p53
456 contributes to sorafenib-mediated miR-30e-3p exosomal secretion by HCC cells.

457 Remarkably, an inverse correlation between tissue and serum miR-30e-3p levels was detected in
458 both DEN-HCC rats and xenograft mice subjected to sorafenib treatment (Pearson's correlation;
459 $R=-0.58$ and $R=-0.88$, respectively) (**Figure S9F, G**). In addition, a negative correlation between
460 miR-30e-3p circulating levels and apoptotic molecules was found in sorafenib-treated HCC rats
461 (Pearson's correlation; $R=-0.82$, -0.65 and -0.51 , respectively) (**Figure S9H-J**) and higher
462 circulating miR-30e-3p levels were detected in sorafenib-resistant group (Student's t-test;
463 $p=0.05$) (**Figure 7I**). Taken together, these correlative findings suggested miR-30e-3p as a
464 potential biomarker of treatment response.

465 We thus tested circulating miR-30e-3p levels in patients with advanced HCC (**Table S2**),
466 assaying sera samples collected before (46 patients, 25 responders and 21 non-responders at two
467 months) and on sorafenib treatment (49 patients, 23 responders and 26 non-responders at the 4

months follow-up assessment). Circulating miR-30e-3p levels did not differ between responders and non-responders (mean circulating miR-30e-3p: 2.24 in R vs 1.96 in NR; unpaired t-test, $p=0.3$, **Figure 7J**) ruling out any predictor role of primary response. Conversely, higher circulating miR-30e-3p was found at the two months follow-up in patients experiencing escape to treatment at the four months TC (circulating miR-30e-3p at two months: 2.13 in responders vs 3.18 in non-responders, unpaired T-test, $p<0.0001$; **Figure 7K**), suggesting its early elevation in acquired drug resistance. In a subgroup of 28 patients (13 responders and 15 non-responders at the four months follow-up), variations of circulating miR-30e-3p were tested over time (basal versus two months follow-up). Even though the cohort is small, preventing any conclusion, an increase in circulating miR-30e-3p levels at the two months assessment was observed in patients developing escape at the subsequent CT-scan (**Figure S9K, L**). Notably, circulating miR-30e-5p levels did not predict sorafenib response neither before nor on treatment (**Figure S9M, N**) further highlighting the specificity of miR-30e-3p variations in acquired resistance to sorafenib. In conclusion, even though these data are very preliminary and circumstantial, the early rising of circulating miR-30e-3p in patients developing sorafenib resistance makes this miRNA appealing for further exploitation, in a field where non-invasive biomarkers are needed.

484 **Discussion**

485 MiRNAs act as tumor suppressors (TS) or oncogenes depending on targets' core, driver gene
486 mutations and tumor microenvironment. Here we report the downregulation of miR-30e-3p in
487 human and rat HCCs and its association with poor prognosis, proliferation, stemness phenotype
488 and drug resistance. The modulation of different biological functions has to be ascribed to the
489 simultaneous targeting of both oncogenes and tumor suppressor genes.

490 Previous studies reported miR-30 as a 'dual' miRNA, with opposite functions in NSCLC and
491 pancreatic cancer [15, 18]. Here, we describe the 'dual' role of miR-30e-3p within the same
492 tumor type, highlighting opposite effects based on different molecular backgrounds and in
493 particular to *TP53* status. On one hand, miR-30e-3p decreased cell proliferation and induced
494 sorafenib sensitization in *TP53* WT HepG2 cells, behaving as a TS miRNA. On the other hand, it
495 induced proliferation, and drug resistance in *TP53*-mutated/deleted HCC cells, behaving as an
496 oncomiR. Indeed, both tumor suppressors (*CDKN1B/p27* and *PTEN*) and oncogenes (*MDM2*,
497 *EpCAM*) were demonstrated to be miR-30e-3p targets. Specifically, miR-30e-3p exerts TS
498 functions in *TP53* WT contexts where *mdm2* targeting is prevalent, leading to p53 pathway
499 activation. On the contrary, tumor-promoting effects take place in non-functional p53 contexts,
500 where p27 and PTEN targeting becomes prevalent. In particular, p27 drives the proliferating
501 phenotype; EpCAM modulates staminal properties, whereas PTEN contributes to drug resistance
502 by triggering AKT activation [46]. Similarly, we previously reported a dual behavior for miR-
503 221 with respect to p53 status, highlighting the importance of molecular characterization when
504 antagomiR strategies are considered [21]. Indeed, both miR-221 and miR-30e-3p modulate the
505 *TP53* axis through *mdm2* direct regulation explaining, at least in part, their opposite activity in
506 different *TP53* contexts.

507 As established for miR-30a and miR-30e-5p [24, 25] here we proved that p53 activates miR-30e
508 transcription. Nevertheless, we observed a negative correlation between primary and mature

509 miR-30e-3p levels, envisaging regulatory events on miRNA biogenesis. Strikingly, mature but
510 not primary miRNA profiles clustered normal and tumor samples into separate nodes, suggesting
511 maturation processes as more relevant than altered transcription for aberrant miRNA expression
512 [47]. Gain-of-function of mutated p53 isoforms hampers miRNA processing machinery leading
513 to miRNA downregulation due to interference with both p68 and p72/82 RNA helicases,
514 impairing Drosha microprocessor activity [23, 48]. Moreover, R273H mutated p53 inhibits miR-
515 30a transcription contributing to its aberrant expression in breast cancer [49]. We observed an
516 association between p53 mutations and miR-30e-3p downregulation, as well as the inhibition of
517 miRNA maturation following Nutlin-3 treatment, suggesting the enrollment of other mdm2
518 targets, such as p53 family members (p63 and p73), as possible regulators of miRNA biogenesis.
519 In this regard, an elegant study by Su and coworkers demonstrated that Dicer1 is a
520 transcriptional target of TAp63 but not of p53 [50]. Our descriptive findings on p53 involvement
521 in miRNA biogenesis led us to speculate that p53 accumulation, as in the case of *MDM2*
522 permanent inhibition by Nutlin-3, might be responsible for the transcriptional inhibition of
523 Dicer1 through saturation of p63/p53-REs, preventing p63-mediated transcription. Dicer1
524 disruption in knockout (KO) mice promoted hepatocarcinogenesis and led to the deregulation of
525 several miRNAs, including all miR-30 family members, suggesting a strict regulation of miR-30
526 maturation by Dicer1 in HCC [51].

527 Rat HCCs, characterized by a *TP53* WT background [42], showed an inverse correlation
528 between miR-30e-3p and tumor size, as well as reduced miR-30e-3p tissue levels in sorafenib
529 resistant tumors, suggesting miR-30e-3p downregulation as a contributor to sorafenib resistance.
530 New molecules, beside sorafenib, were recently registered for advanced HCC [52], however
531 biomarkers predicting treatment outcome are lacking. In the last two decades, miRNAs have
532 emerged as attractive diagnostic, prognostic and therapeutic tools [53, 54] and reliable markers
533 [43]. Decreased miR-30e and miR-223 serum levels were reported in HCC patients irrespective

534 of tumor etiology [55], whereas miR-30e-enriched vesicles decreased mesenchymal properties in
535 cholangiocarcinoma cells [56].

536 Our and other groups reported sorafenib as a trigger for *TP53* activation and demonstrated *TP53*
537 as a key player for exosomal secretion in stressful conditions [45] even though mechanisms
538 governing exosomal secretion remain poorly understood. Here we confirmed the contribution of
539 the p53 pathway to miR-30e-3p exosomal secretion in sorafenib-treated HCC cell lines. In
540 addition, as reported for the TKI gefitinib, sorafenib triggers exosome secretion in HCC cells
541 [44]. Increased miR-30e-3p circulating levels were found in sorafenib resistant rat and human
542 HCCs. In particular, higher miR-30e-3p circulating levels, deriving from exosomal secretion,
543 were associated with subsequent escape to sorafenib. This does not prove a causative effect and
544 may simple represent a bystander event; however, miR-30e-3p role as a non-invasive predictor
545 of treatment escape can be envisaged and should be further investigated in larger patient cohorts.
546

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557

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709 **Figure Legends**

710 **Figure 1. Deregulated expression of miR-30 family in human and rat HCCs.** (A-C) Box plot
711 graphs of miR-30e-3p, miR-30e-5p and miR-30a-3p in human and (D-F) rat tumor and non-
712 tumor (NT) samples or liver cirrhosis (LC). (G-I) Box plot graph of miR-30e-3p, miR-30e-5p
713 and miR-30a-3p in the validation cohort and in normal livers (NL). On the top of each graph is
714 reported the p-value relative to ANOVA, whereas stars represent comparison between groups
715 (Tukey's post hoc test). (J, K) Box plot graphs of mature and primary (pri-miR) miR-30e-3p in
716 wild type (WT) and mutated (MUT) *TP53* HCC specimens. Y-axes report $2^{-\Delta\Delta Ct}$ values
717 corresponding to miRNA levels.

718

719 **Figure 2. *TP53* regulates miR-30e expression in HCC cells.** (A) QPCR analysis of primary
720 miR-30e (pri-miR-30e) and mature isoforms (miR-30e-3p, miR-30e-5p, miR-34a) in *TP53*
721 silenced, (B) *TP53* overexpressing (pCMV-p53) and (C) Nutlin-3 treated HepG2 cells or (D-E)
722 Nutlin-3 treated p53-deleted HepG2 (CRISPR) and p53-null Hep3B cells. (F) QPCR analysis of
723 primary miR-30e and mature isoforms in *TP53* overexpressing Hep3B cells and (G) mutated
724 *TP53* (pBabe-p53-mut) overexpressing HepG2 cells. (A-G) WB and qPCR analyses of *TP53*,
725 *MDM2*, *CDKN1A/p21* in the same settings. U6RNA and β -actin were used as housekeeping
726 genes. QPCR experiments were performed twice in triplicate; p-values from unpaired t-test are
727 shown in each graph. Scr: scramble oligonucleotide; pCMV and pBabe: control vectors; DMSO:
728 vehicle.

729

730 **Figure 3. MiR-30e-3p targets *MDM2* in HCC cells.** (A) MiR-30e-3p hypothetical binding sites
731 in *MDM2* 3'UTR, as identified by Targetscan. Stars represent mutated bases. (B) QPCR analysis
732 of miR-30e-3p in HCC, liver cirrhosis (LC) and normal liver (NL) as well as in HCC cell lines.

733 *TP53* status and relative transcriptional activity are reported in the table below the graph, *TP53*
734 activity has been normalized on that detected in HepG2 cells. (C-E) QPCR and WB analyses of
735 miRNA and *MDM2* expression in miR-30e-3p overexpressing Huh-7, Hep3B and SNU475 cells
736 and (F) miR-30e-3p silenced SNU449 cells. Time after transfection: 24 h. Y-axes represent
737 relative expression levels. U6RNA and β -actin were used as housekeeping genes. (G) Luciferase
738 reporter assay in HepG2 cells co-transfected with wild type (WT) or mutated (MUT) pGL3-
739 MDM2-3UTR vectors and miR-30e-3p or negative control (NC). This experiment was repeated
740 three times in triplicate. (H) P53 reporter assay and WB analysis in miR-30e-3p overexpressing
741 HepG2 and Huh-7 cells. (G, H) Y-axes report relative values with respect to negative controls.
742 NC: pre-miR negative control; NCi: anti-miR negative control; AM-30e-3p: anti-miR-30e-3p.

743

744 **Figure 4. MiR-30e-3p influences cell growth and invasion of HCC cells.** (A, B) Growth
745 curves of miR-30e-3p and negative control (NC) transfected HepG2 cells in basal conditions and
746 following p53 silencing. (C-E) Growth curves of miR-30e-3p transfected Huh-7, Hep3B and
747 SNU475 cells with respect to negative control (NC) cells. (A-E) WB analysis of p53, PTEN,
748 pAKT, p27 and p21 levels normalized on β -actin housekeeping gene. (F) Real time invasion
749 assay of miR-30e-3p stably silenced (MZIP-30e-3p) HepG2 and SNU449 cells with respect to
750 controls (MZIP-shRNA).

751

752 **Figure 5. MiR-30e-3p influences stem cell properties of HCC cells.** (A) Correlation graphs
753 between miR-30e-3p and AFP or EpCAM mRNAs in HCC patients (N=30). Axes report $2^{-\Delta\Delta Ct}$
754 values transformed in a log2 form. (B) QPCR analysis of EpCAM expression in miR-30e-3p
755 overexpressing Huh-7 cells (transient transfection) and miR-30e-3p silenced HepG2 cells (stable
756 infection). Y-axis reports relative gene expression. (C) Luciferase reporter assay in HepG2 cells

757 co-transfected with WT or mutated (MUT) pGL3-EpCAM-3UTR vectors and miR-30e-3p or
758 negative control (NC). **(D-F)** Six-well plate images of clonogenic assay in MIZ-30e-3p and
759 control (MZIP-shRNA) HepG2 and SNU449 cells, as well as in miR-30e-3p overexpressing
760 (pMXs-miR-30e-3p) and control (pMXs-shRNA) Huh-7 cells. Column graphs represent colony
761 count and OD 595. Columns and bars represent average \pm SD values. **(G)** Sphere formation
762 assay in MZIP-30e-3p and control HepG2 cells as well as in pMXs-miR-30e-3p and control
763 (pMXs-shRNA) Huh-7 cells. Average \pm SD values from two independent experiments are
764 shown. **(H)** WB analysis of stemness markers in MZIP-30e-3p HepG2 and pMXs-miR-30e-3p
765 Huh-7 spheres. **(I)** Box plot graph displaying miR-30e-3p expression in patients with or without
766 microvascular invasion (MVI). **(J)** Kaplan-Meier survival curve in HCC patients with high or
767 low miR-30e-3p expression. High or low miRNA values were considered with respect to median
768 value.

769

770 **Figures 6. MiR-30e-3p induces drug sensitization in HepG2 cells.** **(A)** Annexin-V assay in
771 untreated or **(B)** serum deprived (60h) miR-30e-3p overexpressing and control cells. **(C-E)**
772 Annexin-V images, WB analysis and caspase activity assay in miR-30e-3p overexpressing cells
773 following doxorubicin (2.0 μ g/ml, 48h) or **(F-H)** sorafenib treatment (7.5 μ M, 48h). **(I-K)**
774 Annexin-V images, WB analysis and caspase activity assay in p53-silenced miR-30e-3p
775 overexpressing HepG2 cells following sorafenib treatment.

776

777 **Figure 7. Increased miR-30e-3p serum levels associate with sorafenib escape in HCC.** **(A)**
778 Box plot graph displaying miR-30e-3p levels in sorafenib-treated rat HCCs with respect to
779 surrounding livers or **(B)** in responder (R) and non-responder (NR) groups. **(C)** Correlation
780 graph between tissue miR-30e-3p levels and tumor size in sorafenib-treated rat HCCs. **(D)**

781 QPCR analysis of intracellular, (E) extracellular and (F) exosomal miR-30e-3p levels in
782 untreated and sorafenib treated HCC cells. Y-axes report relative values with respect to not-
783 treated cells. DMSO was used as vehicle in untreated cells. Numbers below histograms represent
784 relative cell viability values (%) of sorafenib treated versus untreated cells (48 h) identifying
785 sensitive and resistant cell lines. (G) *TP53* activity assay in sorafenib treated HepG2, SNU449
786 and SNU475 cell lines (5 μ M for 48 h). Y-axis reports relative p53 activity with respect to
787 untreated cells (vehicle: DMSO). (H) QPCR analysis of intracellular, extracellular and exosomal
788 miR-30e-3p levels in scramble and p53-silenced HepG2 cells after sorafenib treatment (5 μ M for
789 48 h). U6RNA and cel-miR-39 were used as housekeeping genes for intracellular and
790 extracellular/exosomal miRNA levels, respectively. Y-axis reports relative values with respect to
791 not-treated cells. (I) Box plot graph displaying circulating miR-30e-3p levels in responder (R)
792 and non-responder (NR) animals. (J) Box plot graph displaying circulating miR-30e-3p levels in
793 serum samples from responder and non-responder HCC patients collected before treatment start
794 and (K) at two-month follow-up. Response/escape to sorafenib was assessed at the subsequent
795 (2-months or 4 months respectively) TC scan. Axes report $2^{-\Delta\Delta C_t}$ values transformed in a log2
796 form.

Figure 1

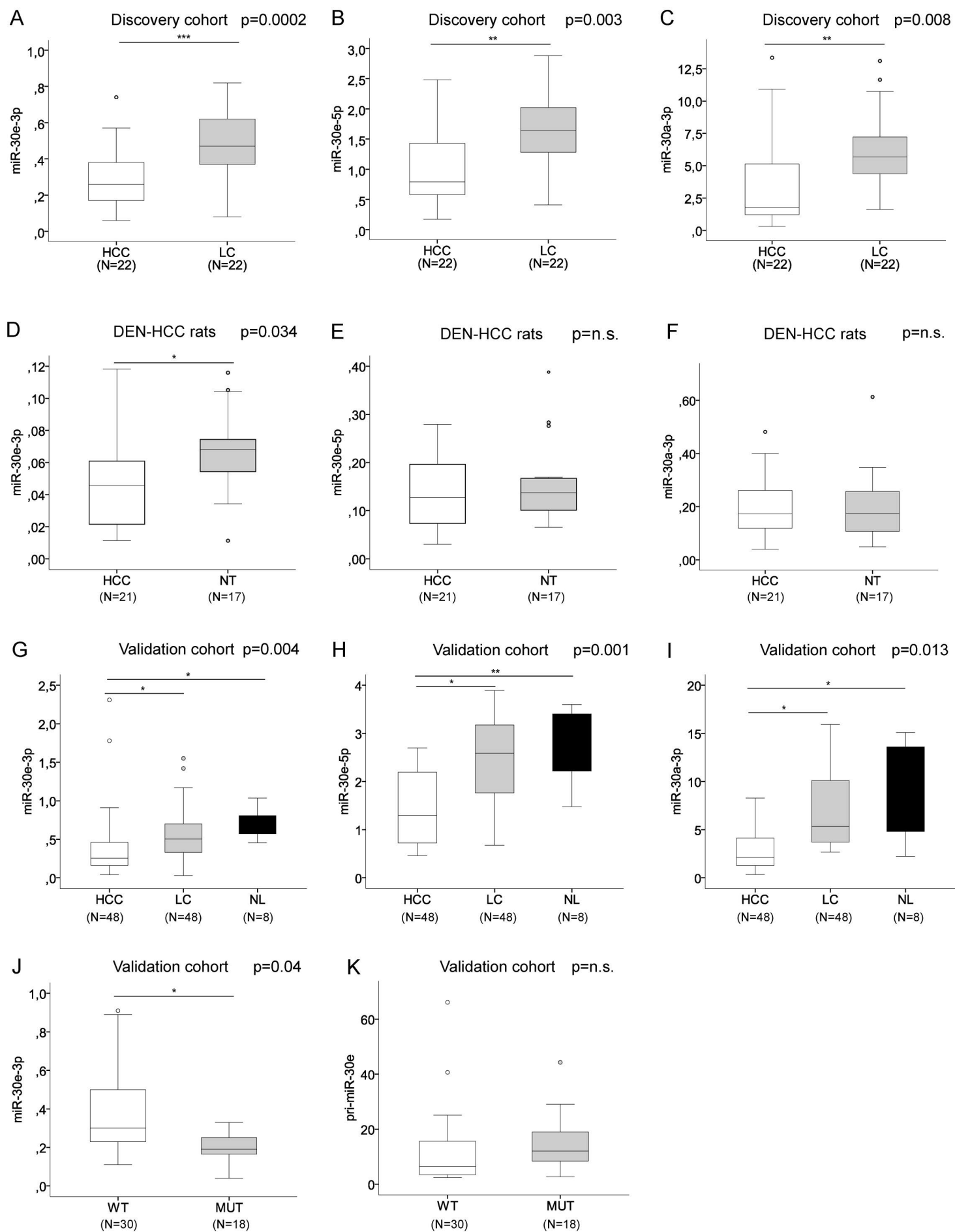


Figure 2

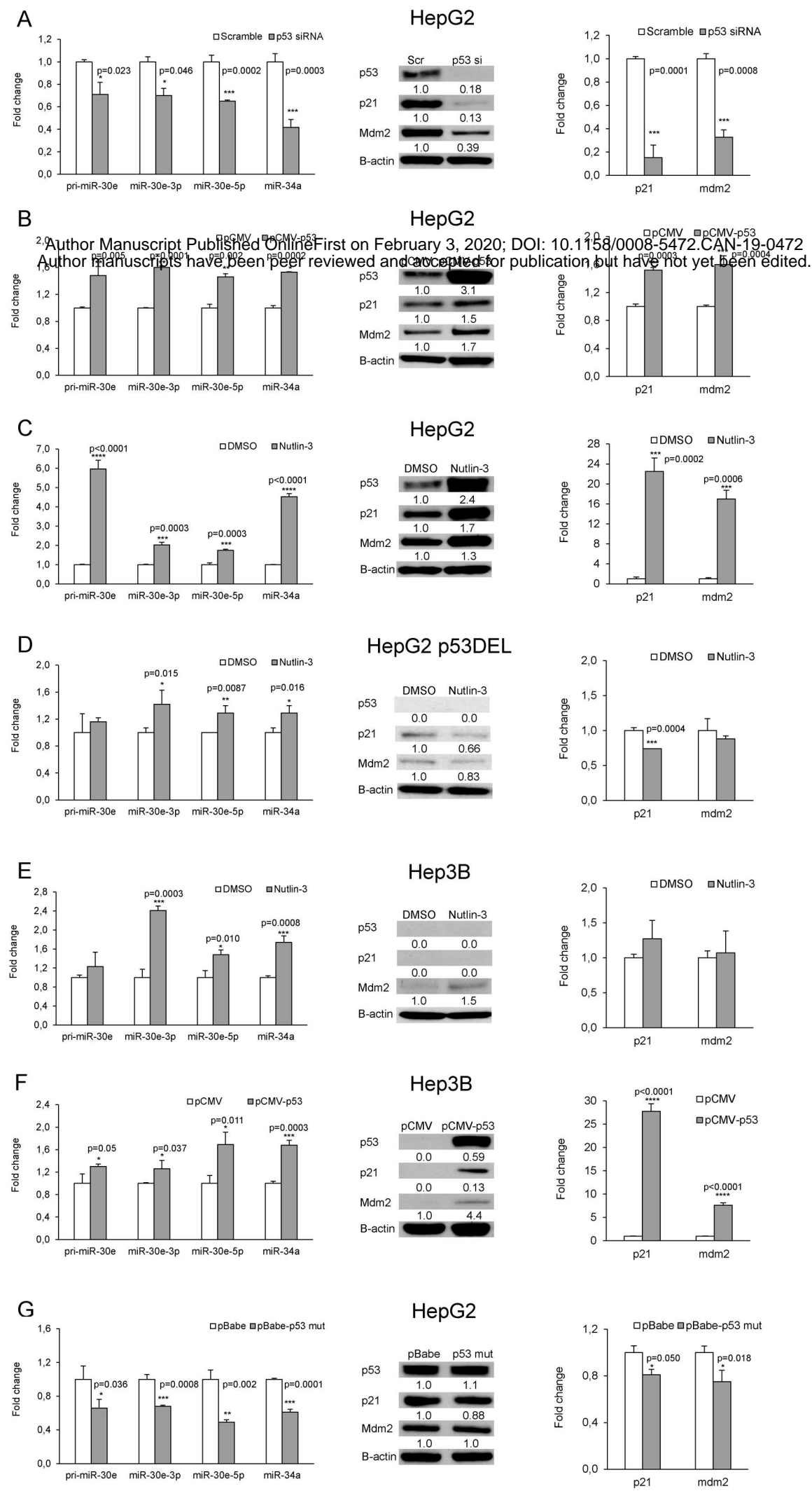


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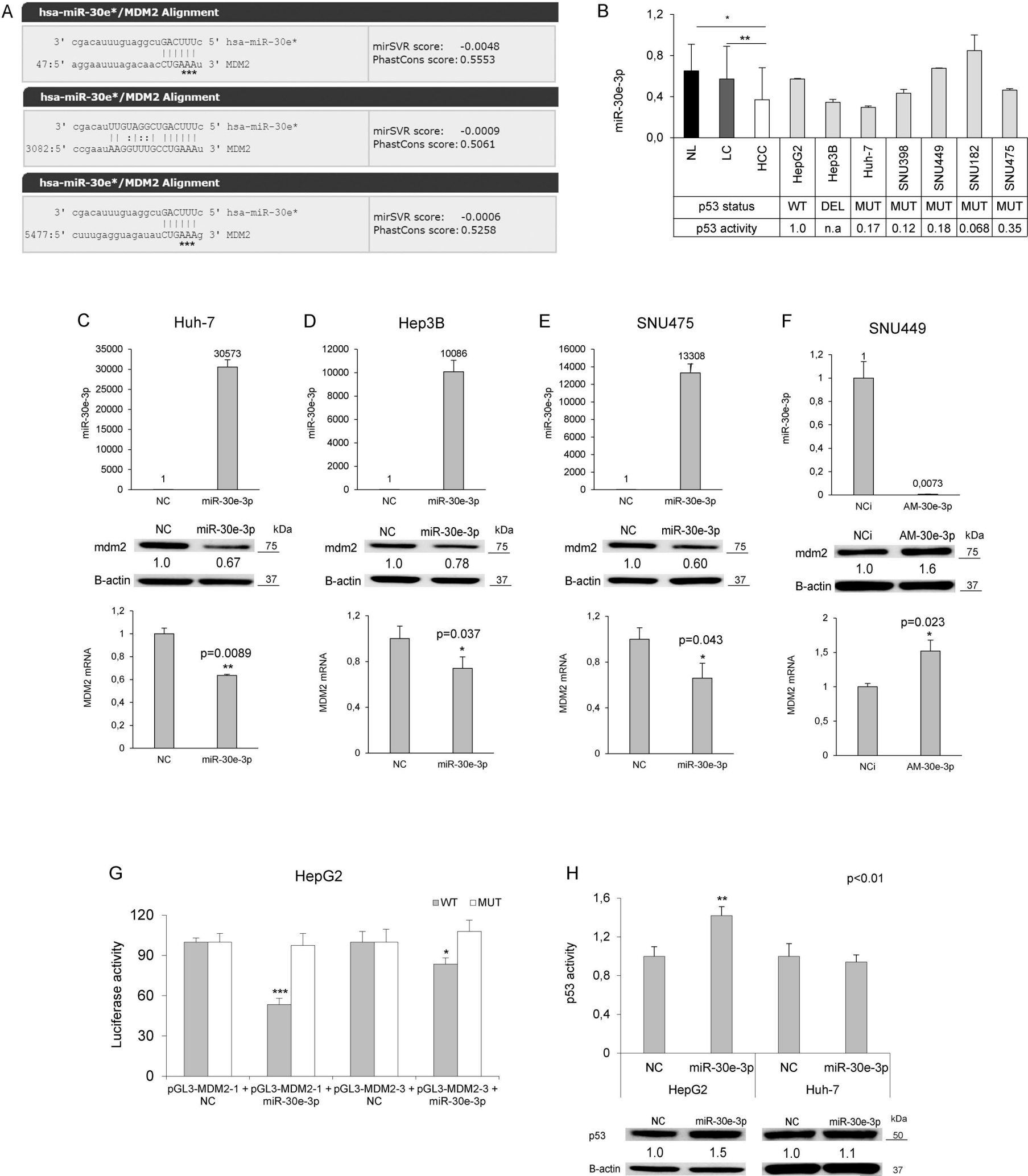
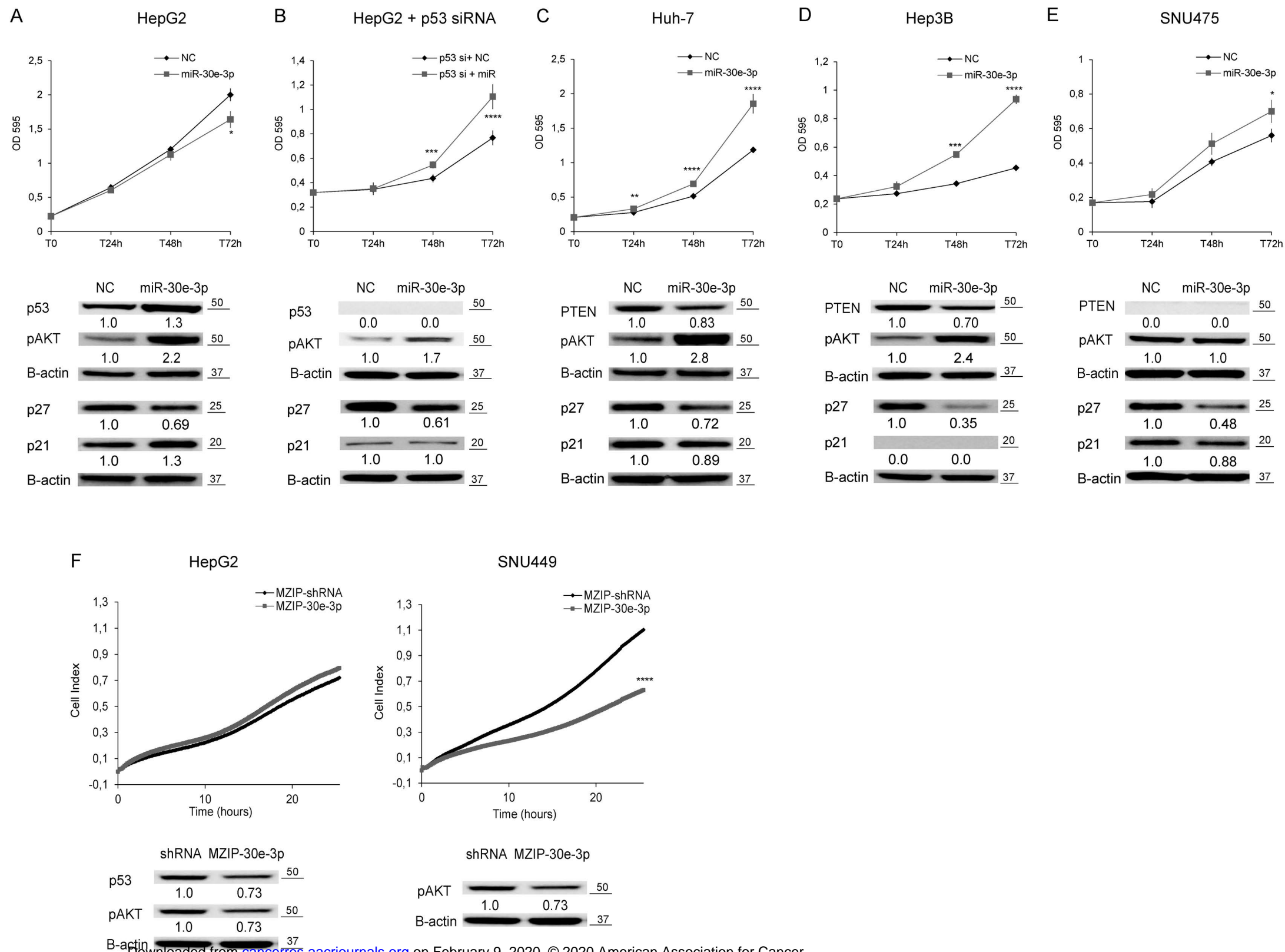


Figure 4



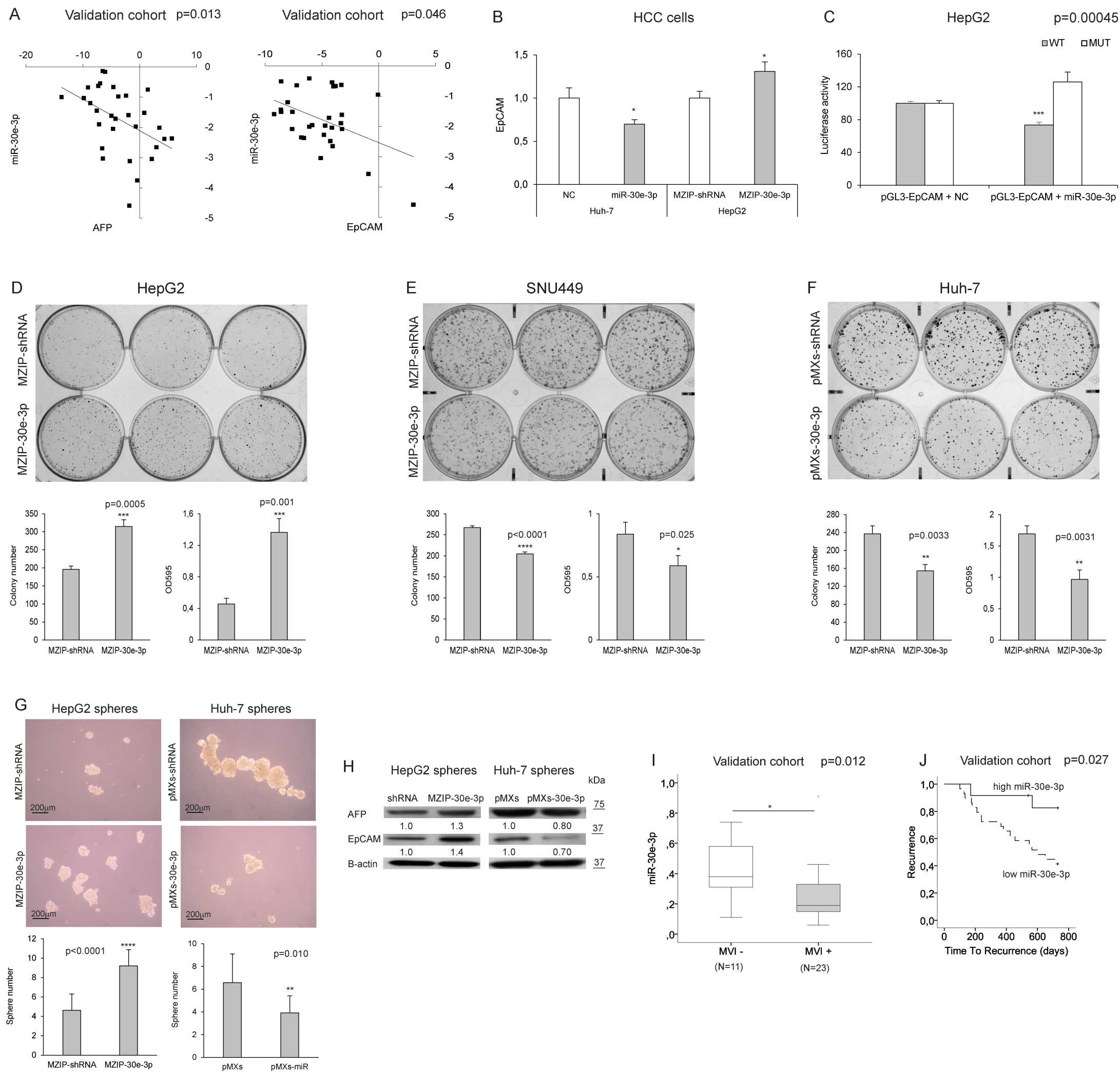


Figure 6

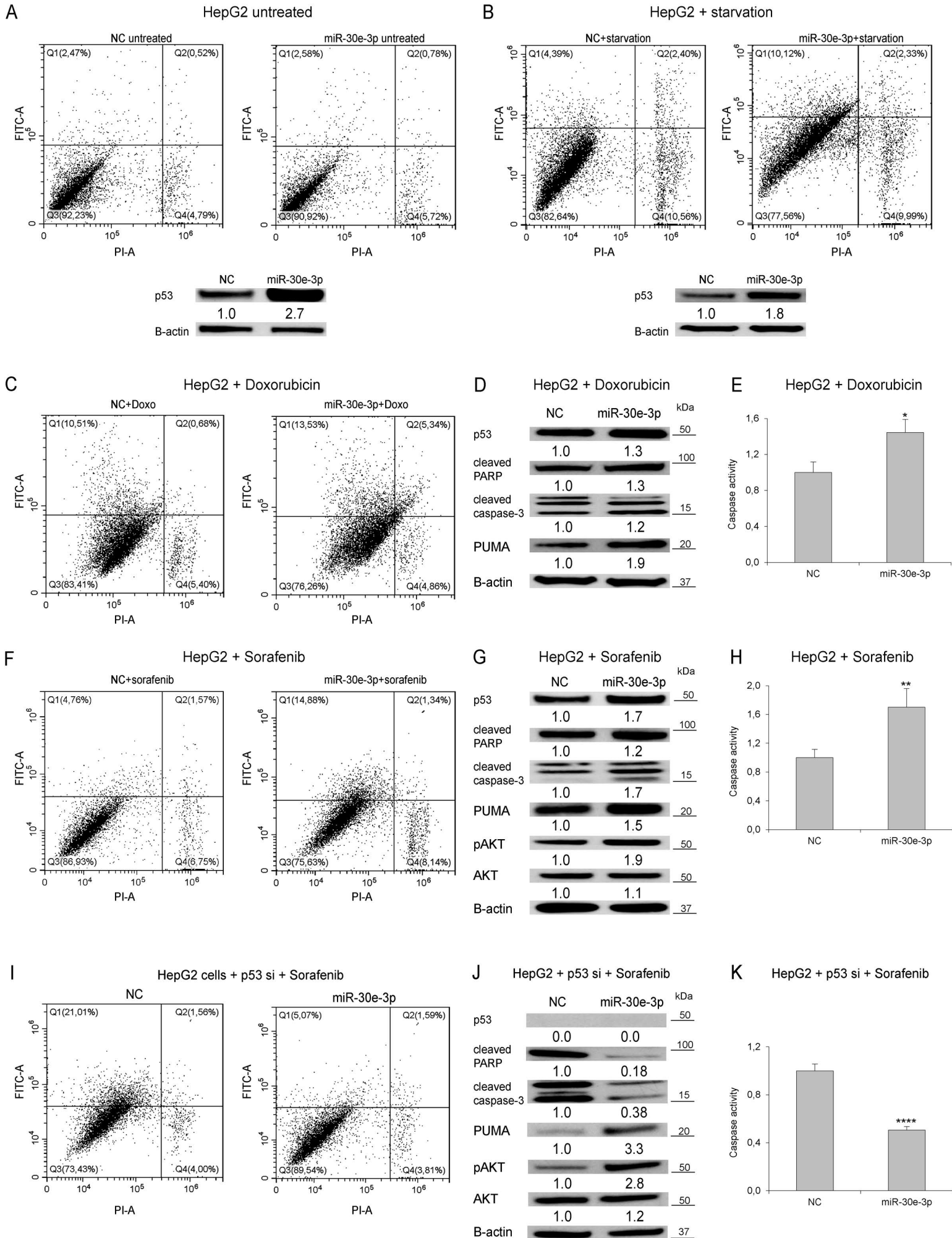


Figure 7

