Activation of v-Myb Avian Myeloblastosis Viral Oncogene Homolog-Like2 (MYBL2)-LIN9 Complex Contributes to Human Hepatocarcinogenesis and Identifies a Subset of Hepatocellular Carcinoma with Mutant p53

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Up-regulation of the v-Myb avian myeloblastosis viral oncogene homolog-like2 B-Myb (*MYBL2*) gene occurs in human hepatocellular carcinoma (HCC) and is associated with faster progression of rodent hepatocarcinogenesis. We evaluated, in distinct human HCC prognostic subtypes (as defined by patient survival length), activation of *MYBL2* and *MYBL2*-related genes, and relationships of p53 status with MYBL2 activity. Highest total and phosphorylated protein levels of MYBL2, E2F1-DP1, inactivated retinoblastoma protein (pRB), and cyclin B1 occurred in HCC with poorer outcome (HCCP), compared to HCC with better outcome (HCCB). In HCCP, highest LIN9-MYBL2 complex (LINC) and lowest inactive LIN9-p130 complex levels occurred. MYBL2 positively correlated with HCC genomic instability, proliferation, and microvessel density, and negatively with apoptosis. Higher MYBL2/LINC activation in HCC with mutated p53 was in contrast with LINC inactivation in HCC harboring wildtype p53. Small interfering RNA (siRNA)-mediated MYBL2/LINC silencing reduced proliferation, induced apoptosis, and DNA damage at similar levels in HCC cell lines, irrespective of p53 status. However, association of MYBL2/LINC silencing with doxorubicin-induced DNA damage caused stronger growth restraint in $p53^{-/-}$ Huh7 and Hep3B cells than in $p53^{+/+}$ Huh6 and HepG2 cells. Doxorubicin triggered LIN9 dissociation from MYBL2 in $p53^{+/+}$ cell lines and increased MYBL2-LIN9 complexes in $p53^{-/-}$ cells. Doxorubicin-induced MYBL2 dissociation from LIN9 led to $p21^{WAF1}$ up-regulation in $p53^{+/+}$ but not in $p53^{-/-}$ cell lines. Suppression of *p53* or *p21*^{WAF1} genes abolished DNA damage response, enhanced apoptosis, and inhibited growth in doxorubicin-treated cells harboring $p53^{+/+}$. *Conclusion:* We show that MYBL2 activation is crucial for human HCC progression. In particular, our data indicate that MYBL2-LIN9 complex integrity contributes to survival of DNA damaged $p53^{-/-}$ cells. Thus, MYBL2 inhibition could represent a valuable adjuvant for treatments against human HCC with mutated p53. (HEPATOLOGY 2011;53:1226-1236)

epatocellular carcinoma (HCC) is the fifth most frequent human cancer, with the highest frequency found in sub-Saharan Africa and far eastern Asia, where hepatitis B virus and hepatitis C virus infections are endemic and food is contaminated with aflatoxin B1.[1-3] HCC is a rapidly fatal disease, with a life expectancy of about 6 months from the time of the diagnosis. Therapies with pharmacological agents or alternative strategies do not substantially improve the prognosis of patients with unresectable HCC.[1-3] This emphasizes the need to investigate the contribution of signaling pathways to tumor development in different HCC subtypes, selected according to their clinical and pathologic features, to identify novel prognostic markers and molecular targets for early diagnosis, chemoprevention, and treatment.

Recent studies on the molecular events leading to cell cycle deregulation in human HCC showed highest up-regulation of iNOS/NF-κB (inducible nitric oxide synthase / nuclear factor-κB) and RAS/ERK (extracellular signal-regulated kinase) signaling, ubiquitination of the ERK inhibitor DUSP1 (dual-specificity phosphatase 1), and deregulation of FoxM1 (Forkhead box M1B) and cell cycle key genes in rapidly progressing human HCC subtype with poorer outcome (HCCP), based on <3 years survival, after partial liver resection, compared to HCC with better outcome (>3 years survival; HCCB).[4-7] Furthermore, the highest ubiquitination and proteasome degradation of cell cycle inhibitors, including P21[WAF1], P27[KIP1], P57[KIP2], P130, RASSF1A (Ras-associated factor 1), and FOXO1 (forkhead box O1) contribute to cell cycle up-regulation and fast progression of HCCP.[8] Interestingly, molecular alterations similar to those found in HCCP occur in chemically induced HCC from rats genetically susceptible to hepatocarcinogenesis, whereas the alterations of HCCB resemble those of HCC induced in genetically resistant rats.[9]

MYBL2 (v-Myb avian myeloblastosis viral oncogene homolog-like2; B-Myb) is a transcription factor belonging to the Myb proto-oncogene family expressed ubiquitously in proliferating cells. Knocking out of MYBL2 leads to early developmental arrest in mice.[10] Numerous observations indicate that MYBL2 together with E2F1-3 regulates the expression of G_2/M genes, including CDC2, and Cyclins A2 and B1 in rat embryonic fibroblasts and human ganglioblastoma.[11] LIN9,

a component of LINC that switches from a transcriptionally repressive complex with p130 or p107 and E2F4, in G_0/G_1, to an active complex in S/G_2, and MYBL2 are both required for transcription of G_2/M genes, Cyclin B1 and Survivin, in undifferentiated embryonic carcinoma cells.[12] Small interfering RNA (siRNA)-mediated MYBL2 knockdown inhibits the expression of CDC2, CCNA2 (encoding Cyclin A2), and TOPA2 (topoisomerase II alpha) in HCC cell lines.[10] MYB family members are implicated in various human cancers, including bone marrow, colon, breast, pancreas, and esophageal malignancies, and melanomas,[10] and cytogenetic analysis revealed that MYBL2 at 20q13.1 is amplified in breast, liver, ovarian carcinomas, and cutaneous T lymphomas.[13-16] However, the role of MYBL2, whose levels are elevated in HCC,[17] has been barely studied in liver cancer. Recent results from our laboratory[18] showed that Mybl2 expression and activation are under genetic control in rodent hepatocarcinogenesis. Indeed, Mybl2 up-regulation was associated with faster growth and progression of premalignant and malignant liver lesions both in chemically treated rats and in a transgenic mouse model. Also, deregulation of G_1-S and G_2-M transition and activation of various genes and pathways related to tumor progression followed MYBL2 gene transfection in human HCC cell lines, substantiating a growth promoting effect of MYBL2 in liver cancer.[18]

In the present study, to further investigate the role of MYBL2 in the progression of human HCC, we comparatively evaluated the changes in the expression and activation of MYBL2 and MYBL2-related genes in HCC prognostic subtypes exhibiting different capacity to grow and progress.[9] Moreover, because recent observations showed a correlation of high MYBL2 levels with a p53 mutant status in human breast cancer,[19] we examined the relationships of p53 status and MYBL2 activity in human HCC.

Materials and Methods

Human Tissue Samples. Six normal livers, 40 surgically resected HCCs, harboring wildtype p53, and 14 HCCs, harboring mutated p53, and corresponding surrounding nontumor liver tissues were used. Mutation status of the p53 gene was assessed as described.[20] When indicated, HCCs with wildtype p53 were divided into two groups based on patient survival length:

HCC with poorer outcome (HCCP; n = 20), and HCC with better outcome (HCCB; n = 20), which were characterized by a shorter (<3 years) or longer (>3 years) survival following liver partial resection, respectively.[21] The clinicopathologic features of HCCs are shown in Supporting Table 1. Liver tissues were kindly provided by Dr. Snorri S. Thorgeirsson (National Cancer Institute, Bethesda, MD). Institutional Review Board approval was obtained at participating hospitals and the National Institutes of Health.

Cell Lines and Treatment. The human HCC cell lines Huh6 and HepG2, harboring wildtype p53 gene, and Huh7 and Hep3B, harboring mutated p53 gene, respectively, were maintained as monolayer cultures in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Cells were plated at 2.0×10^3/well in 96-well plate and grown for 12 hours, then serum-deprived for 24 hours and treated with siRNA against MYBL2, LIN9, p53, or p21 (Santa Cruz Biotechnology, Santa Cruz, CA) following the manufacturer's recommendations. For the induction of DNA damage, cells were treated with 2 μmol/L doxorubicin (Sigma-Aldrich, St. Louis, MO) for 2 hours, washed twice with phosphate-buffered saline (PBS), and returned to normal growth medium for the indicated time periods. All experiments were repeated at least 3 times per each cell line.

Proliferation and Apoptotic Indices. Proliferation and apoptotic indices were determined in human HCC by counting Ki-67-positive cells immunostained with mouse monoclonal anti-Ki67 antibody (clone MIB-1; Dako Deutschland, Hamburg, Germany) and apoptotic figures stained with the ApoTag peroxidase *in situ* apoptosis kit (Millipore, Billerica, MA), respectively, on at least 3,000 hepatocytes. Microvessel density (MVD) was evaluated in HCC slides immunostained with mouse monoclonal anti-CD34 antibody (Vector Laboratories, Burlingame, CA). MVD was expressed as the percentage of the total CD34-stained spots per section area (0.94 mm^2). Activation of MYBL2 in liver tissue slides was assessed by immunohistochemistry using the rabbit monoclonal anti-phospho-MYBL2 antibody (Thr487; Abcam, Cambridge, MA) and was expressed as the percentage of phospho-MYBL2-positive nuclei on 3,000 hepatocytes. Cell viability, apoptosis, and DNA damage of cultured HCC cells were determined by WST-1 Cell Proliferation Reagent, Cell Death Detection Elisa Plus kit (Roche Molecular Biochemicals, Indianapolis, IN), and Damage Quantification Kit (Biovision, Mountain View, CA), respectively, following the manufacturers' instructions.

Random Amplified Polymorphic DNA (RAPD) Analysis. Twenty-two previously designed primers were used to score genomic alterations in human HCCs, and the RAPD reaction was performed as described.[22] Differences from corresponding nontumorous livers were scored in the case of a change in the intensity, absence of a band, or appearance of a new band in HCC. The frequency of altered RAPD profiles was calculated for each liver lesion as reported.[23]

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR). Primers for human *MYBL2* and *RNR-18* genes were chosen with the assistance of the "Assay-on-Demand Products" (Applied Biosystems, Foster City, CA). PCRs and quantitative evaluation were performed as described.[4]

Immunoblot and Immunoprecipitation Analyses. Tissue samples from human liver lesions were processed as reported.[4] Homogenates and nuclear extracts were centrifuged and supernatants were precleared with gamma-binding sepharose beads followed by centrifugation and treatment with normal immunoglobulin G (IgG) control. Protein concentrations were determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine serum albumin as standard. Membranes were probed with specific primary antibodies (Supporting Table 2). Each primary antibody was followed by incubation with horseradish peroxidase-secondary antibody diluted 1:5,000 for 1 hour and then revealed with the Super Signal West Pico Chemiluminescence Substrate Kit (Pierce Chemical, New York, NY). For each protein, densities were calculated by the ImageQuaNT 5.1 software (GE Healthcare, Piscataway, NJ), and normalized to β-actin (Chemicon International, Temecula, CA; dilution 1:20,000) levels.

Statistical Analysis. Data are means $\pm$ standard deviation (SD). Student's t and Tukey-Kramer tests were used to evaluate statistical significance of differences in gene expression, cell viability, and apoptosis. The assumption that the data are sampled from populations that follow Gaussian distribution was tested using the methods of Kolmogorov and Smirnov and D'Agostino and Pearson. Statistical evaluation of nuclear positivity for Ki67 and p-MYBL2 was made by Mann-Whitney U test. Pearson's multiple regression analysis was performed to calculate the correlation coefficient (R), and Cox proportional hazards regression method was used to estimate the predictivity of patients survival. $P < 0.05$ was considered significant.

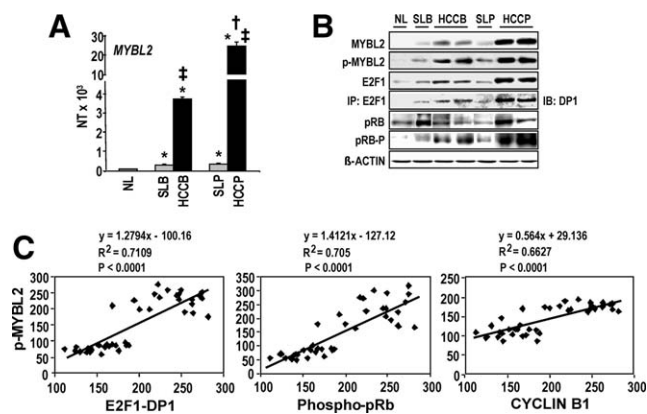Additional methodological details are included in the Supporting Material.

Fig. 1. Expression of MYBL2 and related genes in human neoplastic liver lesions. Liver tumors were divided in two distinct categories based on the length of patient survival after partial liver resection: HCC with better outcome (survival >3 years following partial liver resection; HCCB), and HCC with poorer outcome (survival <3 years; HCCP). (A) MYBL2 mRNA levels were determined by quantitative reverse transcription-PCR. N Target = $2^{-\Delta Ct}$; ΔCt = Ct RNR18-Ct target gene. Data are means (SD) of N target of six normal livers (NL) and 14 HCCB and HCCP, and correspondent surrounding livers (SL) with better or poorer prognosis. Tukey-Kramer test, (A) and (C): *HCC and surrounding subtypes versus NL, at least $P < 0.001$; ‡HCC subtypes versus corresponding surrounding liver, $P < 0.0001$. †Different from HCCB for $P < 0.0001$. (B) Representative western blot analysis of MYBL2, pMYBL2, and MYBL2 related genes in human HCC. Hepatic tissue lysates were subjected to western blotting with specific antibodies. The E2F1-DP1 complex was determined through immunoprecipitation (IP) with the anti-E2F1 antibody and probing the membranes with the anti-DP1 antibody (immunoblot; IB). (C) Relationships between p-MYBL2 levels and E2F1-DP1 complex, phosphorylated pRb, and Cyclin B1 in human HCC.

Results

MYBL2 Is Progressively Up-Regulated During Human HCC Development and Progression.

Increase in MYBL2 messenger RNA (mRNA) levels was detected in surrounding livers irrespective of HCC subtypes and, to a higher extent, in HCC, when compared with normal livers (Fig. 1A). HCCP exhibited mRNA levels 5-fold higher than HCCB. Accordingly, total and phosphorylated (activated) levels of MYBL2 protein were significantly higher in nonneoplastic surrounding livers than in normal livers (Fig. 1B; Supporting Fig. S1A). A further up-regulation of MYBL2 was detected in liver tumors, with the highest levels being found in HCCP (Fig. 1B; Supporting Fig. S1A). Because MYBL2 is a target of the E2F1 transcription factor,[24] we assessed the levels of E2F1 and its interactors in the same sample collection. A similar trend of expression to that of MYBL2 was detected for the levels of total and activated E2F1 (bound to the transcription factor DP1) as well as for those of hyper-phosphorylated/inactivated retinoblastoma protein (pRB), but not for active/hypophosphorylated pRB.

Accordingly, active MYBL2 (p-MYBL2) significantly correlated with E2F1-DP1 and phospho-pRB levels, as well as with the established MYBL2 target, Cyclin B1[12] (Fig. 1C).

Next, we investigated the levels of the LIN Complex (LINC), forming an active complex with MYBL2 in S/G_2 phases of the cell cycle,[12,19,25,26] with regard to Cyclin B1 activation. A progressive increase of MYBL2-Cyclin B1 nuclear complexes (a sign of activated Cyclin B1) and a gradual decrease of p130-Cyclin B1 and E2F4-Cyclin B1 (signs of inactivated Cyclin B1) occurred in nontumorous surrounding liver to HCC, especially in HCCP (Fig. 2A; Supporting Fig. S1B). Furthermore, levels of LINC activation, as determined by LIN9-MYBL2 nuclear complexes, were highest in HCCP. Levels of LIN9-p130 (a sign of LINC inactivation) were instead lowest in HCCP, whereas no differences in total levels of LIN9 were detected (Fig. 2A; Supporting Fig. S1B). Because MYBL2 levels might be negatively modulated by SKP2-(S-phase kinase-associated protein 1-dependent)-degradation,[27] we determined whether an impaired binding of MYBL2 with SKP2 could influence MYBL2 protein levels in HCC. A progressive increase of MYBL2-SKP2 complexes as well as of ubiquitinated levels of MYBL2 was detected from nontumorous surrounding livers to HCC (Fig. 2B,C), reaching the highest levels in HCCP, implying that MYBL2 up-regulation is not due to its impaired degradation in human HCC.

Correlation of MYBL2 Activation with Clinicopathological Parameters in Human HCC.

Due to the different behavior of MYBL2 expression in HCCs with different survival rate, we evaluated the relationship of MYBL2 activation with clinicopathologic features to explore the prognostic role of MYBL2 in human HCC. The genomic instability, as assessed by RAPD analysis, proliferation index, and microvessel density values correlated with MYBL2 activation/phosphorylation (Fig. 3). In contrast, an inverse correlation of MYBL2 activation with apoptosis was found (Fig. 3). The determination of the predictivity of patients' survival on the basis of p-MYBL2 level showed an estimated hazard ratio of 1.03 (95% confidence interval [CI]: 1.01-1.06; $P = 0.013$, n = 40), indicating a low but significant influence of p-MYBL2 expression levels on survival rate. No significant correlation between MYBL2 activation and other clinicopathological parameters, including etiology, sex, age, presence of cirrhosis, α-fetoprotein, tumor size, and grading was found.
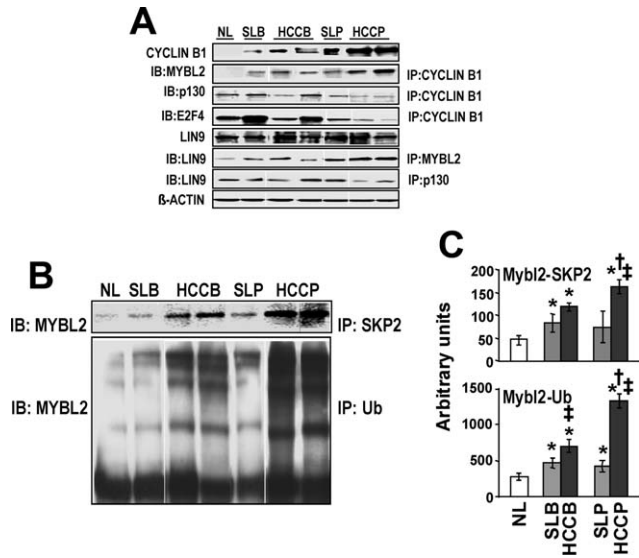
Fig. 2. (A) Representative western blot analysis of Cyclin B1 and its complexes with MYBL2, p130, and E2F4, and of LIN9 and its complexes with MYBL2 and p130 in human HCC. Liver tumors were divided in two distinct categories based on the length of patient survival after partial liver resection: HCC with better outcome (survival > 3 years following partial liver resection; HCCB), and HCC with poorer outcome (survival <3 years; HCCP). Hepatic tissue lysates were subjected to western blotting with specific antibodies. The Cyclin B1 and LIN9 complexes were determined through immunoprecipitation (IP) of nuclear protein extracts with the anti-Cyclin B1 and LIN9 antibodies, respectively, and probing the membranes with the anti-MYBL2, anti-p130, or anti-E2F1 antibodies (immunoblot; IB). (B) Representative immunoprecipitation of MYBL2/SKP2 complexes and of MYBL2 ubiquitinated in human hepatocellular carcinoma. (C) Chemiluminescence analysis of MYBL2/SKP2 complexes and ubiquitinated MYBL2 showing the mean (SD) of six normal livers (NL), 20 hepatocellular carcinomas with better outcome (HCCB), and 20 HCCs with poorer outcome (HCCP), and corresponding surrounding livers (SL). Tukey-Kramer test: *HCC and surrounding subtypes versus normal liver, at least $P < 0.001$; ‡HCC subtypes versus corresponding surrounding liver, at least $P < 0.05$. †Different from HCCB for $P < 0.0001$.

Activation of MYBL2 and LINC in Liver Tumors with Mutated p53. Because it has been recently demonstrated that high levels of MYBL2 positively correlate with a p53 mutant status in human breast cancer,[19] we tested whether the same applies for HCC. Immunohistochemical assessment of Ki67 and p-MYBL2 showed that nuclear MYBL2 increased, concurrently with Ki67, in all surrounding liver and HCC subtypes (Fig. 4; Supporting Fig. S2) with respect to normal liver. No differences occurred between surrounding liver subtypes, whereas the percentages of both p-MYBL2 and Ki67-positive nuclei were significantly higher in HCC than surrounding liver counterparts, and progressively increased from HCCB to HCCP and HCCp53$^{-/-}$. Next, we compared the levels of MYBL2 and LINC between HCC harboring wildtype (n = 40) or mutated (n = 14) *p53* gene by immunoblotting and immunoprecipitation. Ten of 14

HCC with mutated p53 (71.4%) behaved as the poorer outcome patients group. Strikingly, the whole set of markers of MYBL2 and LINC activation (total and activated levels of MYBL2, Cyclin B1, LIN9-MYBL2, and MYBL2-Cyclin B1 complexes) were significantly higher in HCC with a mutant *p53* gene (Fig. 5; Supporting Fig. S3). On the other hand, markers of LINC inactivation (LIN9-p130, p130-Cyclin B1, and E2F4-Cyclin B1 complexes) were significantly higher in HCC harboring wildtype p53 gene (Fig. 5; Supporting Fig. S3). Thus, the present findings indicate that strongest up-regulation of MYBL2 and LINC expression is characteristic of HCC with mutant p53.
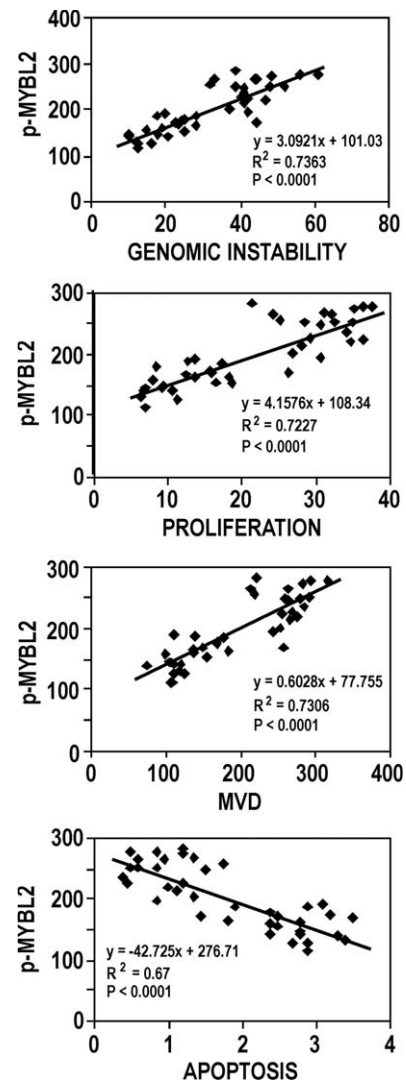

Fig. 3. Relationships between p-MYBL2 levels and genomic instability as assessed by RAPD, proliferation index (percentage of Ki67-positive cells), MVD, and apoptotic index (percentage of apoptotic bodies), in human HCCs.
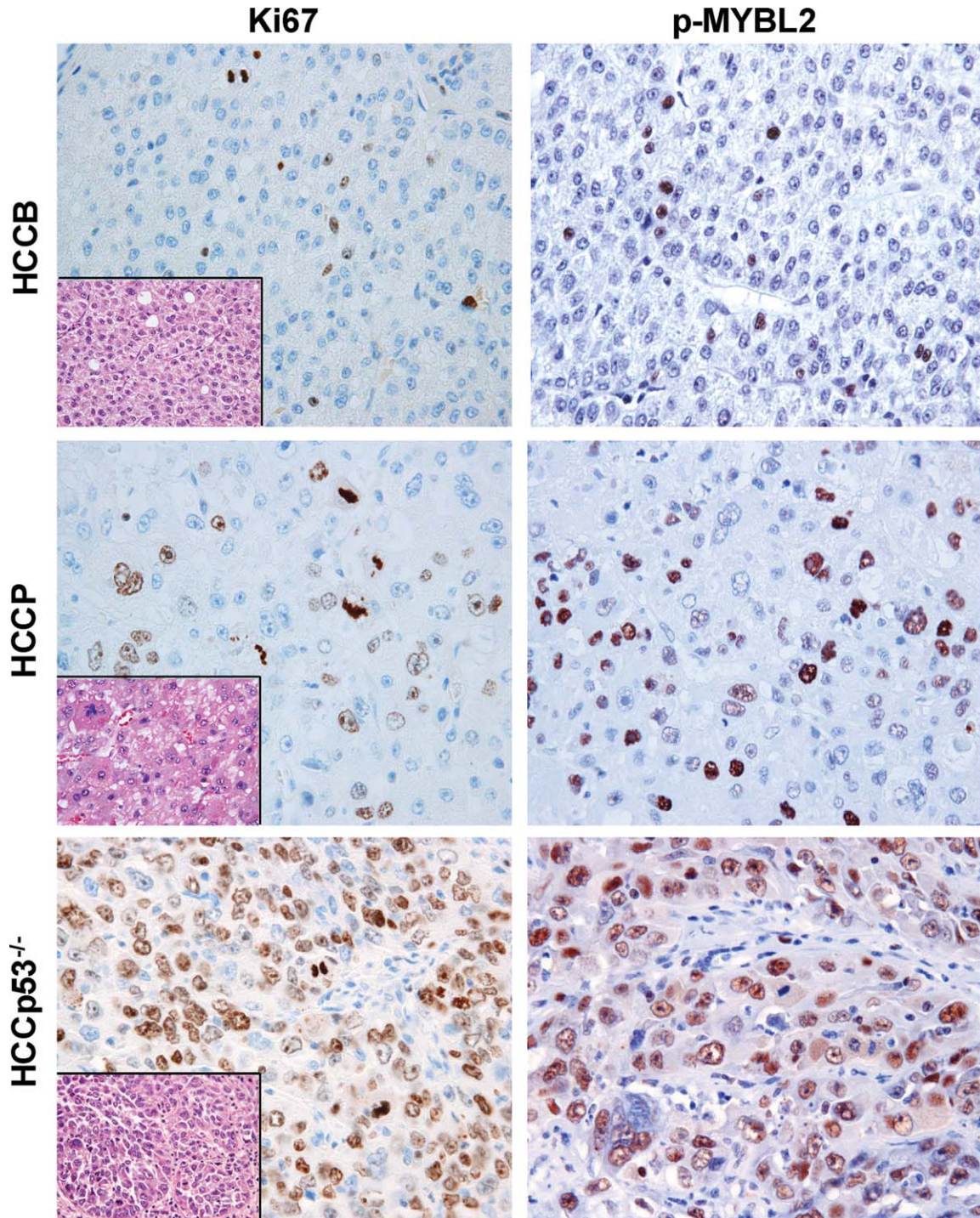
Fig. 4. Representative Ki67 and phosphorylated-MYBL2 (p-MYBL2) immunohistochemical patterns in HCC subtypes with wildtype p53 and better (HCCB) or poorer (HCCP) outcome, and HCC with mutated p53 (HCCp53$^{-/-}$). Insets: Hematoxylin-eosin stained lesions. Note the progressive and parallel increase of both Ki67 and p-MYBL2 nuclear immunoreactivity from SL to HCCp53$^{-/-}$ Original magnification ×200.

Suppression of MYBL2/LINC Increases Sensitivity to Doxorubicin of Human HCC Cells with Mutant p53. We further investigated the role of MYBL2 and LINC in human HCC cell lines by assessing the consequences of *MYBL2* and *LIN9* inactivation by way of siRNA. For this purpose, we selected four HCC cell lines expressing *MYBL2* and *LIN9* genes: Huh6, HepG2 (harboring wildtype p53), Huh7, and Hep3B (harboring mutant p53). In basal condition, the highest overall levels of MYBL2 expression were detected in HepG2 and Hep3B cells, whereas no significant differences in LIN9 levels were found among the four
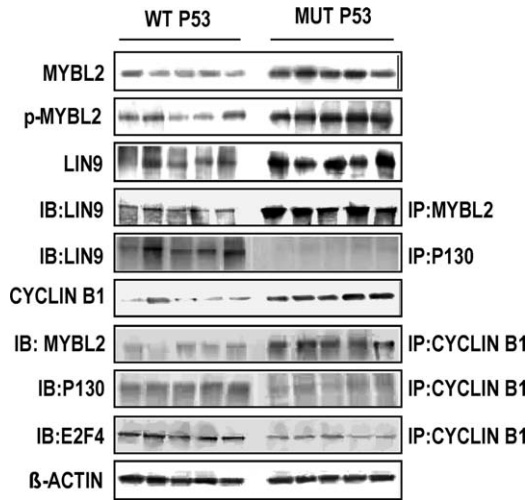
Fig. 5. Representative western blot analysis of Mybl2, p-Mybl2, and Lin9, and Cyclin B1 and its complexes with MYBL2, p130, and E2F4, and of LIN9 and its complexes with MYBL2 and p130 in human HCC harboring either wildtype (WT) or mutated (MUT) *p53* gene. Hepatic tissue lysates were subjected to western blotting with specific antibodies. The Cyclin B1 and LIN9 complexes were determined through immunoprecipitation (IP) of nuclear protein extracts with the anti-Cyclin B1 and LIN9 antibodies, respectively, and probing the membranes with anti-MYBL2, anti-p130, or anti-E2F1 antibodies (immunoblot; IB).

cell lines (Supporting Fig. S4). However, the levels of activated/phosphorylated MYBL2 and the activation of LINC (as assessed by MYBL2-LIN9 complexes) were higher in Huh7 and Hep3B cells and, consequently, inactivation of LINC (as determined by LIN9-p130 complexes) was more elevated in HepG2 and Huh6

cells (Supporting Fig. S4). Specific siRNA showed the same efficiency in the inhibition of *MYBL2* and *LIN9* in the four cell lines irrespective of the p53 status (Supporting Fig. S5). *MYBL2* suppression had no effect on LIN9 levels, whereas silencing of *LIN9* resulted in decreased expression of MYBL2 in Huh6 (p53 wildtype) and Huh7 (p53 mutated) cells (Supporting Fig. S5). Analogous results were obtained when *MYBL2* and *LIN9* siRNA experiments were performed in HepG2 (p53 wildtype) and Hep3B (p53 mutated) cells (not shown).

MYBL2 suppression reduced proliferation, induced apoptosis (Fig. 6), and increased DNA damage (Supporting Fig. S6) at similar levels in the four cell lines. However, when silencing of *MYBL2* was associated with treatment with the DNA damaging agent doxorubicin, very strong growth restraint, elevated apoptosis (Fig. 6), and massive DNA damage (Supporting Fig. S6) occurred only in p53 mutant cell lines. A similar, synergistic effect restraining mostly the growth of HCC cell lines with mutant p53 was detected when doxorubicin treatment was coupled to the silencing of the *LIN9* gene (Supporting Fig. S7).

At the molecular level, doxorubicin treatment did not affect MYBL2 and LIN9 levels in any cell line, irrespective of p53 status (Fig. 7; Supporting Fig. S8). Doxorubicin induced binding of p130 to LIN9 and gradual dissociation of MYBL2 from LIN9 in p53 wildtype Huh6 (Fig. 7; Supporting Fig. S8) and
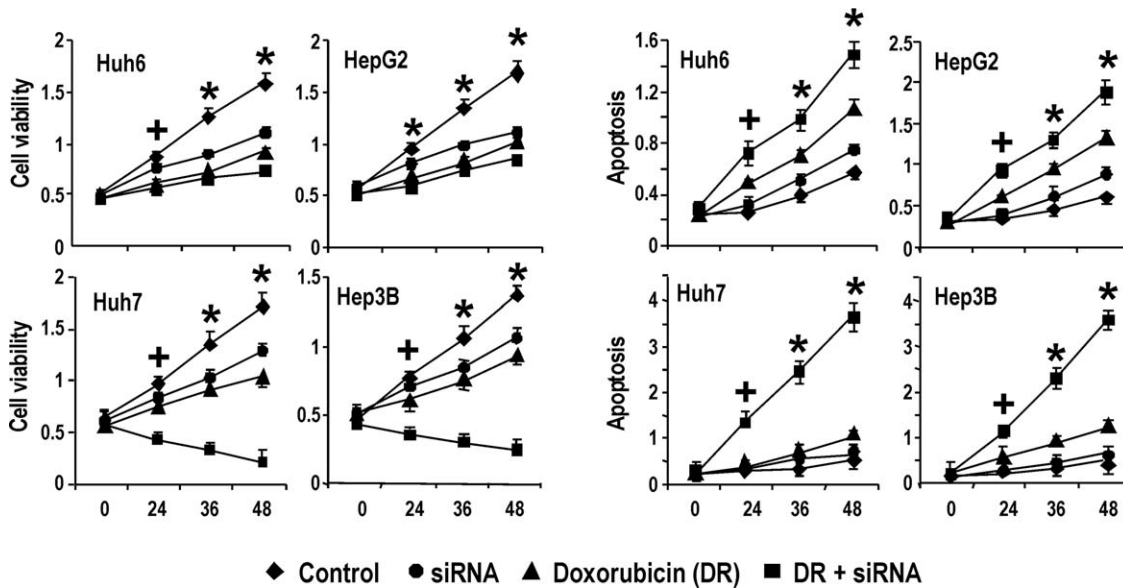
Fig. 6. Effect of the modulation of MYBL2 expression on cell viability and apoptosis of HuH6 and HepG2 human HCC cell lines, harboring wildtype *p53*, and Huh7 and Hep3B, harboring mutated *p53*, respectively. Cells were transfected with 500 ng of *MYBL2* or *LIN9* siRNA or scramble oligonucleotide (SC), and harvested 24, 36, and 48 hours after transfection. Data are means (SD) of three independent experiments conducted in triplicate. Tukey-Kramer test: *siRNA, doxorubicin, and siRNA plus doxorubicin versus control (SC), at least $P < 0.01$; +doxorubicin and siRNA plus doxorubicin versus control, at least $P < 0.05$. Doxorubicin versus siRNA plus doxorubicin, $P < 0.001$ at 24, 36, and 48 hours.

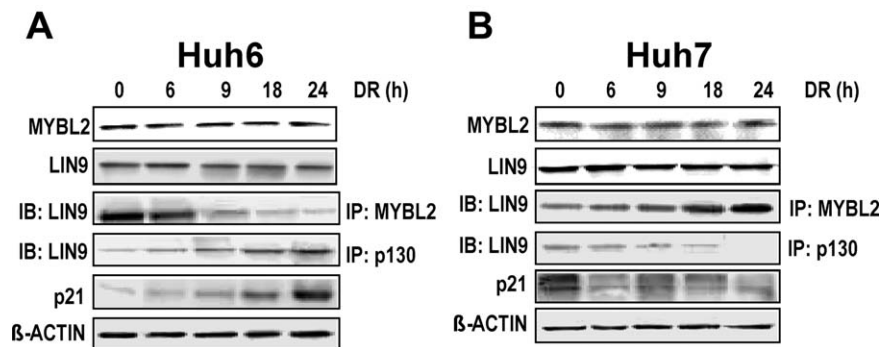Fig. 7. Levels of the LINC in human hepatoma cell lines, harboring wildtype or mutated *p53*, following treatment with the DNA damage inducer doxorubicin. Representative western blot analysis of MYBL2, Lin9, LIN9 complexes with MYBL2 or p130 proteins, and of p21 after treatment with doxorubicin in Huh6 (wildtype *p53*; A), and Huh7 (mutated *p53*; B) cells. Equivalent results were obtained in HepG2 and Hep3B cells (not shown). HCC cells were treated with 2 μmol/L doxorubicin (DR) for 2 hours. At 6, 9, 18, and 24 hours after treatment, nuclear extracts were subjected to immunoprecipitation (IP) with either anti-MYBL2 or p130 antibodies and the membranes probed with the anti-LIN9 antibody (immunoblot, IB), or were subjected to western blotting with the anti-p21 antibody.

HepG2 cells (not shown). In striking contrast, MYBL2-LIN9 binding was not reduced but paradoxically increased by doxorubicin administration in p53 mutant Huh7 cells (Fig. 7; Supporting Fig. S8) and HepG3 cells (not shown). Notably, the dissociation of MYBL2 from LIN9 induced by doxorubicin treatment was paralleled by up-regulation of the p53 target, p21^{WAF1}, in p53 wildtype, but not in p53 mutant cell lines (Fig. 7; Supporting Fig. S8), suggesting that doxorubicin treatment induces a DNA repair program by way of the p53/p21^{WAF1} axis in wildtype p53 cell lines.

To directly test whether the p53/p21^{WAF1} cascade is necessary for the DNA damage response as well as for the association of p130 with LIN9 in p53 wildtype cell lines, HepG2 and Huh6 cells treated with doxorubicin were subjected to either *p21*^{WAF1} or *p53* silencing (Fig. 8; Supporting Fig. S9). Of note, suppression of either *p53* or *p21*^{WAF1} genes abolished the DNA damage response in the two cell lines (as indicated by the increased DNA damage) and enhanced apoptosis, leading to a remarkable growth restraint (Fig. 8). Altogether, these results suggest that the integrity of the MYBL2-LIN9 complex is required for survival of HCC cells with mutant p53 in the presence of DNA damage.

Discussion

The present body of data indicates that MYBL2 plays a critical role in human hepatocarcinogenesis. Indeed, in liver tissue specimens, MYBL2 is ubiquitously and progressively induced from nontumorous surrounding liver to HCC, with the highest increase being detected in the most aggressive HCCP.
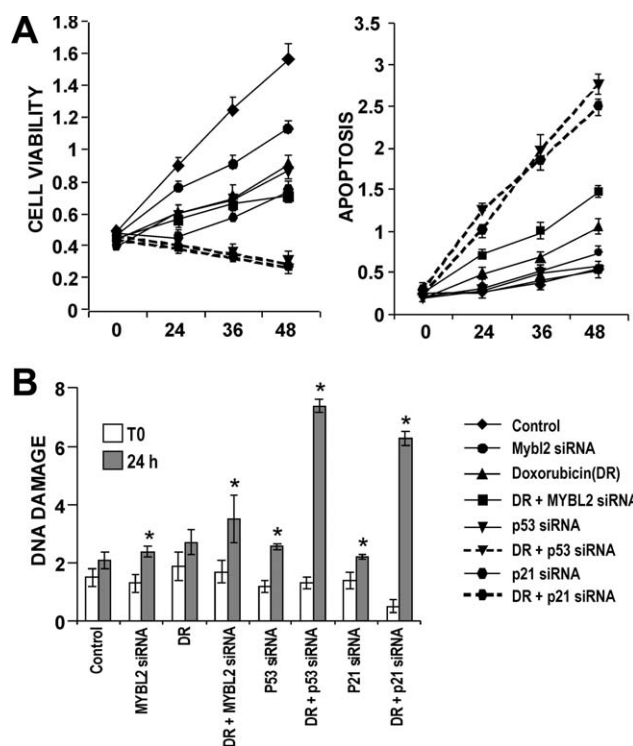

Fig. 8. Effect of suppressing either *p21*^{WAF1} or *p53* gene on viability, apoptosis, and DNA damage in human HepG2 and HuH6 HCC cell lines treated with doxorubicin. HepG2 cells were treated for 2 hours with 2 μmol/L doxorubicin (DR) and transfected with 500 ng of *p21*, *p53* siRNA, or scramble oligonucleotide (SC). DNA damage is expressed as absolute number of apurinic (AP) sites per 10^5 base-pairs. Data are means (SD) of three independent experiments conducted in triplicate. Equivalent results were obtained in Huh6 cells (not shown). Tukey-Kramer test: cell viability, MYBL2-, p53-, p21-siRNA, and DR versus Control (SC), $P < 0.001$ at 24-48 hours. MYBL2-, p53-, p21-siRNA versus DR + MYBL2-, DR + p53-, and DR + p21-siRNA, $P < 0.001$ at 24-48 hours. Apoptosis, MYBL2-siRNA versus Control, $P < 0.001$ at 36 and 48 hours. DR versus Control, $P < 0.001$ at 24-36 hours. MYBL2-, p53-, p21-siRNA versus DR + MYBL2-, DR + p53-, and DR + p21-siRNA, $P < 0.001$ at 24-48 hours. DNA damage, *24 hours versus T0, $P < 0.001$.

Moreover, in agreement with the recent observation that forced MYBL2 up-regulation in human HCC cell lines leads to increase in G_1-S and G_2-M transition,[18] significant correlations of p-MYBL2 levels with its target Cyclin B1,[12] involved in G_2/M phase of cell cycle, and active E2F1 and hyperphosphorylated/inactive pRB, involved in G_1/S transition, were found in HCC. This finding suggests a clear contribution of MYBL2 to HCC growth and progression. A crucial function of MYBL2 in liver cancer is also supported by the observation that p-MYBL2 levels in HCC directly correlate with genomic instability, proliferation index, and tumor microvessel density, and inversely correlate with the apoptotic index. These findings envisage the possibility that p-MYBL2 is a prognostic marker for HCC.

Functional activity of MYBL2 is mostly mediated by its complex with LIN9 (LINC). LINC has been found to be important in the transcriptional regulation of G_2-M genes,[25-29] and to be implicated in the cell cycle arrest produced by DNA damage.[30,31] DNA damage is associated with a switch of MYBL2 from an active complex with LIN9 to a repressive complex with p130 (Supporting Fig. S8), p107, or E2F4.[29] This switch in LINC composition contributes to the transcriptional repression of G_2-M genes. According to our results, total and activated/phosphorylated levels of MYBL2, Cyclin B1, LIN9-MYBL2, and MYBL2-Cyclin B1 complexes are significantly higher in HCC harboring a mutant $p53$ gene, whereas LINC inactivation occurs in HCC with wildtype $p53$ gene. This observation indicates the existence of a strongest up-regulation of MYBL2 and LINC in HCC with mutant $p53$. In agreement with our findings, it has been shown that MYBL2 fails to dissociate from LINC in breast cancer cells harboring mutant $p53$.[19] The presence of mutant $p53$ results in only slight increase in proliferation for HCC with respect to fast-growing HCCP harboring wildtype $p53$ (Supporting Fig. S2). Accordingly, in basal conditions, MYBL2-LIN9 complex (LINC activation) was only slightly higher, and LIN9-p130 complex (LINC inactivation) slightly lower in Huh7 and Hep3B cells (harboring mutant $p53$), than in HepG2 and Huh6 cells (harboring wildtype $p53$). On the other hand, MYBL2 and LIN9 silencing by siRNA had roughly the same inhibitory effect on viability, and enhancing effect on apoptosis in HCC cells, independently of $p53$ status. This indicates that elevated levels of MYBL2, Cyclin B1, LIN9-MYBL2, and MYBL2-Cyclin B1 complexes, characteristic of HCCP harboring wildtype $p53$, allow maximum activation of G_2-M transition and proliferation rate.

One of the major roles of $p53$ is the preservation of genetic stability by preventing genome mutation. Once activated in response to cellular stress or DNA damage, $p53$ attempts to impede further damage by inducing cell cycle arrest to allow either DNA repair or apoptosis.[31,32] In cells harboring wildtype $p53$, the inhibition of G_1 kinases by p21[WAF1] leads to accumulation of dephosphorylated p130, a condition necessary for formation of the LIN9-p130 complex. Cell cycle control by p21[WAF1] cannot occur in cells harboring mutated $p53$ and, consequently, LIN9-p130 complex cannot be formed in response to DNA damage in these cells. The latter display instead high levels of MYBL2-LIN9 (Fig. 5; Supporting Figs. S3 and S10).

Cells harboring mutations in the $p53$ gene cannot arrest in the G_1 phase of the cell cycle. As a consequence of the requirement of p53 and p21[WAF1] to sustain G_2 arrest, after DNA damage,[19,33] p53^{−/−} cells can enter into mitosis. Indeed, when DNA damage was induced by doxorubicin, silencing of either $MYBL2$ or $LIN9$ led to a much higher inhibition of proliferation and stimulation of apoptosis in cell lines harboring mutant $p53$ with respect to cell lines with wildtype $p53$ (Fig. 6; Supporting Figs. S6, S7, and S10). Furthermore, the observation that doxorubicin treatment induces LIN9 binding to p130 (leading to the dissociation of MYBL2-LIN9 complex) and up-regulation of the $p53$ target, $p21^{WAF1}$, only in $p53$ wildtype cell lines, suggests that doxorubicin treatment induces a DNA repair program by way of the p53/ p21[WAF1] axis in wildtype $p53$ cell lines. On the other hand, these data indicate that, in the presence of DNA damage, the integrity of the MYBL2-LIN9 complex is required for survival of HCC cells with mutant $p53$. In agreement with this hypothesis, the inhibition of either $p53$ or $p21^{WAF1}$ gene expression enhanced DNA damage and apoptosis, leading to a remarkable growth restraint, in cell lines with wildtype $p53$ (Fig. 8; Supporting Figs. S9, S10). Altogether, these findings indicate that MYBL2 promotes the survival of $p53$ mutant HCC cells when treated with a DNA damaging agent. This agrees with the observation that chicken DT40 cells, lacking MYBL2, are highly sensitive to DNA damage.[34] In $p53$ mutant cells, up-regulation of MYBL2 could allow cell cycle to resume and cells with damaged DNA to proliferate, thus contributing to genomic instability and HCC progression.

A variety of recent studies showed that the $p53$ gene plays a major role in hepatocarcinogenesis.[21,35,36] $p53$ is at the crossroads of the cellular stress response pathway.[35,36] $p53$ mutation has been detected in 23%-26% of human HCC related to hepatitis B or hepatitis C

virus infection,[37,38] whereas a high amount of a *p53* point mutation occurs, as an early event, in HCC related to exposure to aflatoxin B1.[36] In addition, *p53* is the most frequently mutated gene in human cancer, with a mutation rate over 50%. Therefore, our findings are relevant in light of ongoing combination chemotherapy and underline the importance of assessing the *p53* status for therapeutic approaches aimed at inhibiting the expression or function of MYBL2 in cancer, not restricted to liver tumors.

In conclusion, our findings strongly suggest a crucial role of MYBL2 in human HCC development and progression, and the possibility that MYBL2 acts as a putative prognostic marker for HCC. A dual role is played by MYBL2, upon DNA damage, in HCC cells harboring a wildtype or a mutated *p53*. In HCC cells with wildtype *p53*, MYBL2 induces a DNA repair program by way of the p53/p21^{WAF1} axis. In HCC cells with mutant *p53* instead, the integrity of the MYBL2-LIN9 axis is required for cell survival in the presence of DNA damage, and the inhibition of MYBL2 and/or LIN9 leads to cell death. Our results underline the importance of assessing the *p53* status for combined therapeutic approaches in which the inhibition of the expression or function of MYBL2, in *p53* mutated cells, could represent a valuable adjuvant.

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