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 p21WAF1 up-regulation in p53+/+ but not in p53−/− cell lines. Suppression of p53 or p21WAF1 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)

Abbreviations: DUSP1, dual-specificity phosphatase 1; ERK, extracellular signal-regulated kinase; FOXM1, forkhead box M1; FOXO1, forkhead box O1; HCC, hepatocellular carcinoma; HCCB, HCC with better outcome; HCCP, HCC with poorer outcome; IKK, inhibitor of NF-kB kinase; iNOS, inducible nitric oxide synthase; LIN, LIN complex; MVD, microvessel density; MYBL2, v-Myb avian myeloblastosis viral oncogene homolog-like2; NF-kB, nuclear factor-kB; pRB, retinoblastoma protein; QRT-PCR, quantitative reverse transcription polymerase chain reaction; RAPD, random amplified polymorphic DNA; RASSF1A, Ras-associated factor 1A; SKP2, S-phase kinase-associated protein 1.

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Hepatocellular 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. 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. 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).<sup>4,7</sup> Furthermore, the highest ubiquitination and proteasome degradation of cell cycle inhibitors, including P21<sup>WAF1</sup>, P27<sup>KIP1</sup>, P57<sup>KIP2</sup>, P130, RASSF1A (Ras-associated factor 1), and FOXO1 (forkhead box O1) contribute to cell cycle up-regulation and fast progression of HCCB.<sup>9</sup> 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.<sup>9</sup>

MYBL2 (v-Myb avian myeloblastosis viral oncogene homolog-like 2; 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.<sup>10</sup> Numerous observations indicate that MYBL2 together with E2F1-3 regulates the expression of G2/M genes, including CDC2, and Cyclins A2 and B1 in rat embryonic fibroblasts and human ganglioblastoma.<sup>11</sup> LIN9, a component of LINC that switches from a transcriptionally repressive complex with p130 or p107 and E2F4, in G0/G1, to an active complex in S/G2, and MYBL2 are both required for transcription of G2/M genes, Cyclin B1 and Survivin, in undifferentiated embryonic carcinoma cells.<sup>12</sup> Small interfering RNA (siRNA)-mediated MYBL2 knockdown inhibits the expression of CDC2, CCNA2 (encoding Cyclin A2), and TOP2A (topoisomerase II alpha) in HCC cell lines.<sup>10</sup> MYB family members are implicated in various human cancers, including bone marrow, colon, breast, pancreas, and esophageal malignancies, and melanomas, and cytogenetic analysis revealed that MYBL2 at 20q13.1 is amplified in breast, liver, ovarian carcinomas, and cutaneous T lymphomas.<sup>13-16</sup> However, the role of MYBL2, whose levels are elevated in HCC, has been barely studied in liver cancer. Recent results from our laboratory<sup>18</sup> 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 G1-S and G2-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.<sup>18</sup>

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.<sup>9</sup> Moreover, because recent observations showed a correlation of high MYBL2 levels with a p53 mutant status in human breast cancer,<sup>19</sup> 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.<sup>20</sup> 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. 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 x 10^5/well in 96-well plate and grown for 12 hours, respectively. Cells were treated with 2 mol/L doxorubicin (Sigma-Aldrich, St. Louis, MO) for 2 hours, washed twice with phosphate-buffered saline (PBS), and 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 then 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. 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.

**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). PCR and quantitative evaluation were performed as described.

**Immunoblot and Immunoprecipitation Analyses.** Tissue samples from human liver lesions were processed as reported. 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 1:20,000 levels. Membranes were then incubated 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 ± 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.
**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,\(^2^4\) 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 hyperphosphorylated/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\(^1^2\) (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,\(^1^2,19,25,26\) 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,\(^2^7\) 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, \(\alpha\)-fetoprotein, tumor size, and grading was found.
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, 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.
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
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
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, p21WAF1, 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/p21WAF1 axis in wildtype p53 cell lines.

To directly test whether the p53/p21WAF1 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 p21WAF1 or p53 silencing (Fig. 8; Supporting Fig. S9). Of note, suppression of either p53 or p21WAF1 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.
Moreover, in agreement with the recent observation that forced MYBL2 up-regulation in human HCC cell lines leads to increase in G1-S and G2-M transition, significant correlations of p-MYBL2 levels with its target Cyclin B1, involved in G2/M phase of cell cycle, and active E2F1 and hyperphosphorylated/inactive pRb, involved in G1/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 G2-M genes, and to be implicated in the cell cycle arrest produced by DNA damage. 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. This switch in LINC composition contributes to the transcriptional repression of G2-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. 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 G2-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. In cells harboring wildtype p53, the inhibition of G1 kinases by p21WAF1 leads to accumulation of dephosphorylated p130, a condition necessary for formation of the LIN9-p130 complex. Cell cycle control by p21WAF1 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 G1 phase of the cell cycle. As a consequence of the requirement of p53 and p21WAF1 to sustain G2 arrest, after DNA damage, 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, p21WAF1, only in p53 wildtype cell lines, suggests that doxorubicin treatment induces a DNA repair program by way of the p53/p21WAF1 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 p21WAF1 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. 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. p53 is at the crossroads of the cellular stress response pathway. p53 mutation has been detected in 23%-26% of human HCC related to hepatitis B or hepatitis C.
virus infection,\textsuperscript{37,38} whereas a high amount of a $p53$ point mutation occurs, as an early event, in HCC related to exposure to aflatoxin B1.\textsuperscript{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 $p53/p21^{\text{WAF1}}$ 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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