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Forkhead box M1B is a determinant of rat susceptibility to hepatocarcinogenesis and sustains ERK activity in human HCC

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ABSTRACT

Background and aim: Previous studies indicate unrestrained cell cycle progression in liver lesions from hepatocarcinogenesis-susceptible Fisher 344 (F344) rats and a block of G_1–S transition in corresponding lesions from resistant Brown Norway (BN) rats. Here, the role of the Forkhead box M1B (*FOXM1*) gene during hepatocarcinogenesis in both rat models and human hepatocellular carcinoma (HCC) was assessed.

Methods and results: Levels of FOXM1 and its targets were determined by immunoprecipitation and real-time PCR analyses in rat and human samples. FOXM1 function was investigated by either FOXM1 silencing or overexpression in human HCC cell lines. Activation of FOXM1 and its targets (Aurora Kinose A, Cdc2, cyclin B1, Nek2) occurred earlier and was most pronounced in liver lesions from F344 than BN rats, leading to the highest number of Cdc2–cyclin B1 complexes (implying the highest G_2–M transition) in F344 rats. In human HCC, the level of FOXM1 progressively increased from surrounding nontumorous livers to HCC, reaching the highest levels in tumours with poorer prognosis (as defined by patients' length of survival). Furthermore, expression levels of FOXM1 directly correlated with the proliferation index, genomic instability rate and microvessel density, and inversely with apoptosis. FOXM1 upregulation was due to extracellular signal-regulated kinase (ERK) and glioblastoma-associated oncogene 1 (GLI1) combined activity, and its overexpression resulted in increased proliferation and angiogenesis and reduced apoptosis in human HCC cell lines. Conversely, FOXM1 suppression led to decreased ERK activity, reduced proliferation and angiogenesis, and massive apoptosis of human HCC cell lines.

Conclusions: FOXM1 upregulation is associated with the acquisition of a susceptible phenotype in rats and influences human HCC development and prognosis.

Human hepatocellular carcinoma (HCC) is one of the most common tumours worldwide, accounting for ~500 000 deaths annually.[1–3] Better understanding of the molecular mechanisms underlying hepatocarcinogenesis may hasten the identification of novel molecular markers for HCC progression and the development of new diagnostic and therapeutic strategies.

Studies on rodent hepatocarcinogenesis demonstrated a polygenic predisposition to HCC.[4] Numerous hepatocarcinogenesis susceptibility and resistance loci control rodent HCC development, suggesting a genetic predisposition with a major locus and various low-penetrance genes, at play in different subsets of population.[4] This genetic model is in keeping with human HCC epidemiology.[5] Analysis of the effector mechanisms of susceptibility genes indicates that early preneoplastic liver lesions, induced by chemical carcinogens, grow and progress autonomously to HCC only in susceptible but not in resistant rat strains.[4] Thus, the comparative evaluation of molecular alterations during hepatocarcinogenesis in rats susceptible or resistant to this disease may help in elucidating the mechanisms responsible for human liver malignant transformation. We previously showed overexpression of c-*myc*, *cyclin D1*, *cyclin E*, *cyclin A* and *E2f1* genes associated with pRb (retinoblastoma protein) hyperphosphorylation in neoplastic nodules and HCCs developed in Fisher 344 (F344) rats, susceptible to hepatocarcinogenesis, but not in corresponding lesions from resistant Brown Norway (BN) rats.[4 6] These observations imply a deregulation of G_1 and S phases in liver lesions of susceptible rats, and a block of G_1–S transition in the lesions of the resistant strain, explaining their limited ability to progress. A similar deregulation in cell cycle proteins occurs in human and murine hepatocarcinogenesis, implying the existence of similar molecular mechanisms of hepatocarcinogenesis across species.[2–4 6 7] Recent data indicate that unrestrained cell cycle progression may depend on activation of the Ras–mitogen-activated protein kinase (MAPK) cascade in rodent[8] and human HCC.[9] Indeed, higher expression of extracellular signal-regulated kinase (ERK) was detected in neoplastic nodules and HCC of susceptible F344 rats, whereas ERK was slightly induced in corresponding lesions from the resistant BN strain.[8] Similarly, ERK activity was elevated in human HCC, and highest in tumours with a poor prognosis, implying its crucial role in tumour progression.[9] In particular, we found that ERK achieves unrestrained activity in human HCC by triggering degradation of its specific inhibitor, dual-specificity phosphatase 1 (DUSP1), via the synergistic activity of S-phase kinase-associated protein 2 (SKP2), CDC28 protein kinase 1b (CKS1) and ERK.[10] However, the ERK downstream targets underlying unrestrained cell cycle progression in human and rat HCC remain elusive. A major ERK effector is the Forkhead box M1B (FOXM1) transcription factor, whose overexpression occurs in various experimental and human tumours.[11–17] FOXM1 promotes proliferation through its ability to influence various cell cycle phases. Indeed, FOXM1 triggers the activation of SKP2/CKS1 ubiquitin ligase, which targets

p21[Cip1] and p27[Kip1] proteins for degradation during the G_1–S transition.[11–17] Furthermore, FOXM1 transcriptionally activates CDC2, CYCLIN B1, AURORA KINASE A (AURKA), AURORA KINASE B, SURVIVIN, NIMA-related kinase 2 (NEK2) and centromere protein A and B, thus allowing G_2–M progression.[11–17] In the mouse liver, FOXM1 depletion results in block of proliferation and resistance to hepatocarcinogenesis.[18–20] However, the role of FOXM1 in rat liver with respect to genetic susceptibility to hepatocarcinogenesis and its prognostic significance in human HCC have not been elucidated to date. Here, we evaluated the influence of susceptible and resistant genotypes to hepatocarcinogenesis on FOXM1 expression in neoplastic rat liver lesions differently prone to progress to more malignant stages. Next, we assessed the correlation between FOXM1 deregulation and patients' clinicopathological features and prognosis in human HCC. Our results indicate that FOXM1 expression is highest in liver neoplastic lesions from susceptible F344 rats and is associated with a poor prognosis in human HCC. Furthermore, FOXM1 triggers degradation of the ERK inhibitor DUSP1 via transcriptional activation of SKP2 and CKS1, thus determining a positive loop reinforcing ERK activity in human HCC.

MATERIALS AND METHODS

Animals and treatments

F344 and BN rats (Charles-River-Italia, Calco, Italy) were fed, housed, and treated according to the "resistant hepatocyte" protocol,[21] consisting of a 150 mg/kg intraperitoneal dose of diethylnitrosamine followed by 15 days of feeding a 0.02% 2-acetylaminofluorene-containing hyperprotein diet, with a partial hepatectomy at the midpoint of this feeding regime.[6] Preneoplastic liver (4–6 weeks after initiation), early nodules (mostly clear/eosinophilic cell nodules; 12 weeks), neoplastic nodules (32 weeks) and HCCs (57–60 weeks) were collected. Animals received human care, and study protocols were in compliance with the National Institutes of Health guidelines for use of laboratory animals.

Human tissue samples

Six normal livers, 26 HCCs with poor (HCCP) and 32 HCCs with better (HCCB) prognosis, with <3 and >3 years survival following partial liver resection, respectively,[7] and corresponding surrounding non-tumour livers were used. Patients' clinicopathological features are shown in Supplementary table 1. Liver tissues were kindly provided by Dr Snorri S Thorgeirsson (NCI, Bethesda, Maryland, USA). Institutional Review Board approval was obtained at participating hospitals and from the National Institutes of Health.

Cell lines and treatments

HuH6, HLE (exhibiting high FOXM1 levels) and SNU-182 (exhibiting low FOXM1 expression) human HCC cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). A total of 2.0×10^6 HuH6 and HLE cells in 10 cm dishes were incubated for 12 h at 37°C before transfection with small interfering RNA (siRNA) duplexes specific to human FOXM1, ERK2 and glioblastoma-associated oncogene 1 (GLI1), as described.[17 22 23] siRNAs and scramble oligonucleotides (final concentration 100 nmol/l) were transfected using the siPORT NeoFX system (Ambion, Austin, Texas, USA). For transient transfection experiments, SNU-182 cells were transfected with ERK2 (wild-type) in a pUSEamp plasmid (Millipore, Millerica,

Massachusetts, USA), and FOXM1 and GLI1 (wild-type) cDNA in a pCMV6-XL vector (OriGene Technologies, Rockville, Maryland, USA) following the manufacturer's protocol. For flow cytometry experiments, 8×10^5 HuH6 cells in 10 cm dishes were cultured for 12 h in Optimem medium (Invitrogen, Carlsbad, California)/10% FBS, and then for 48 h in medium with 0.2% FBS. After synchronisation (T_0), the cells were transfected with siRNA anti-FOXM1 or scramble oligonucleotide as above, incubated for an additional 48 h and then collected, washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol at 4°C for 12 h. Fixed cells were incubated with RNase A and propidium iodide prior to flow cytometric analysis (BD FACSCalibur, Becton Dickinson, Italia, Buccinasco, Milan, Italy).

Proliferation and apoptotic indices

Proliferation was determined in human HCC by counting Ki-67-positive cells, and apoptotic figures were stained with the ApoTag peroxidase in situ apoptosis kit (Millipore), on at least 3000 hepatocytes. Viability and apoptosis of cell lines in vitro were determined by the WST-1 Cell Proliferation Reagent and the Cell Death Detection Elisa Plus kit (Roche Diagnostics, Indianapolis, Indiana), respectively.

Quantitative reverse transcription-PCR (QRT-PCR)

Primers for rat FOXM1 and RNR-18 genes were chosen with the assistance of the "Assay-on-DemandProducts" (Applied Biosystems, Foster City, California, USA). PCRs and quantitative evaluation were performed as described.[24]

Immunoprecipitation analysis

Tissue samples from human livers, early nodules (12 weeks after initiation), nodules and HCCs from F344 and BN rats were processed as reported.[24] Membranes were probed with specific primary antibodies (Supplementary table 2). Cyclin B1–Cdc2 complexes were determined through immunoprecipitation with the anti-cyclin B1 antibody and probing the membranes with the anti-Cdc2 antibody. Bands were quantified in arbitrary units by the ImageMaster Total LabV1.11 software, and normalised to actin levels.

Erythropoietin (EPO) and vascular endothelial growth factor α (VEGFα) assays

HuH6, HLE and SNU-182 cell culture medium was collected, centrifuged to remove cellular debris and stored at −70°C until assayed for EPO and VEGFα by ELISA following the supplier's protocol (R&D Systems, Minneapolis, Minnesota, USA). Data were expressed in mU/100 μg and in pg/μg proteins per well for EPO and VEGFα, respectively.

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.[25] Differences from corresponding non-tumorous 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.[26]

Chromatin immunoprecipitation (ChIP) analysis

ChIP was performed using the ChIP Assay Kit (Millipore) following the manufacturer's protocol. Rabbit polyclonal anti-FOXM1 (Santa Cruz Biotechnology, Santa Cruz, California,
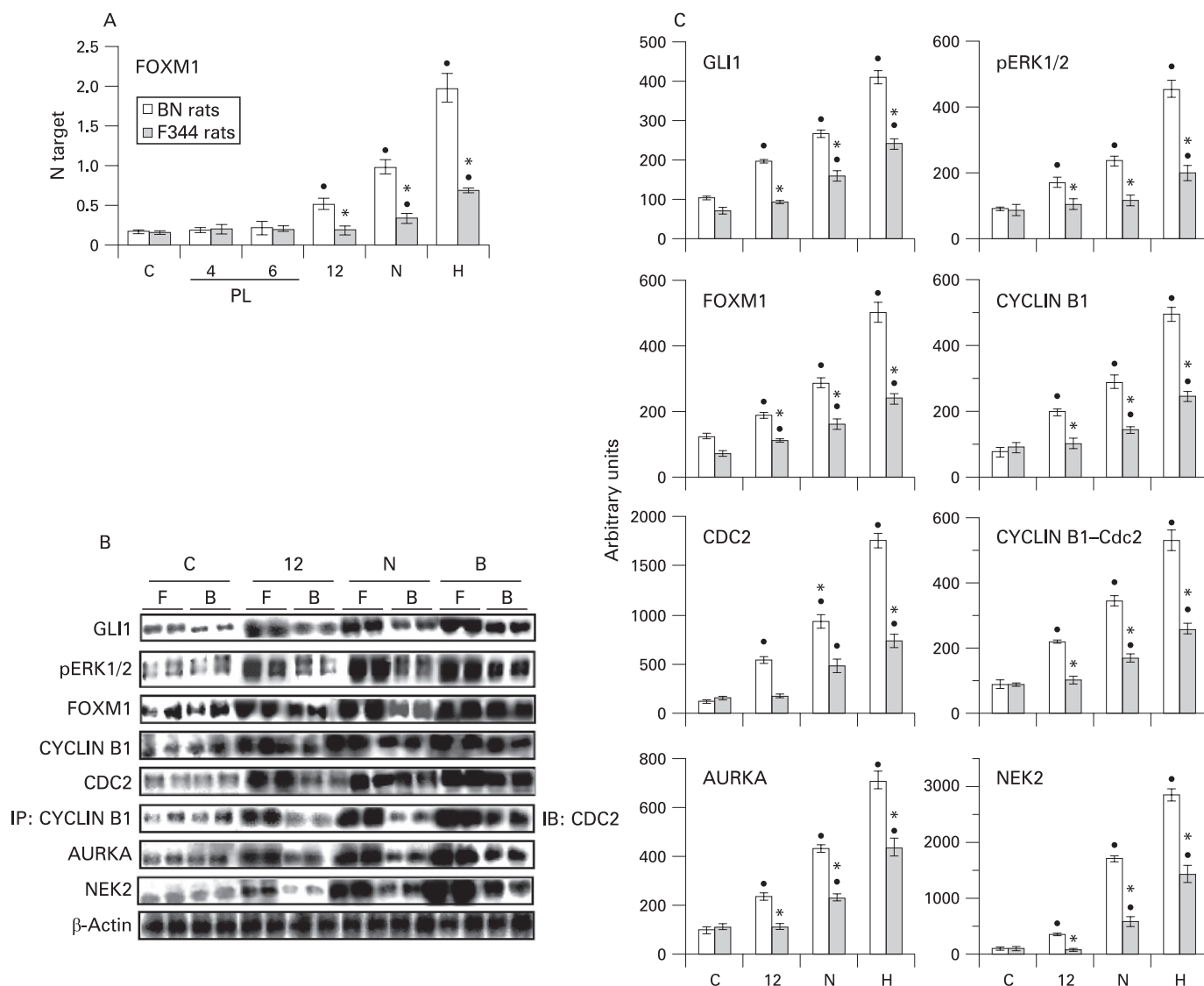
Figure 1 Expression of *FOXM1* (Forkhead box M1B) and related genes in preneoplastic and neoplastic liver of Fisher 344 (F344) and Brown Norway (BN) rats. Preneoplastic liver (4 and 6 weeks after initiation), early nodules (12 weeks), neoplastic nodules and hepatocellular carcinomas (HCCs) were induced in rats treated according to the ''resistant hepatocyte'' model. (A) *FOXM1* mRNA levels were determined by quantitative reverse transcription-PCR. N Target $= 2^{-\Delta Ct}$; $\Delta Ct = Ct$ RNR18–Ct target gene. Data are means (SD) of N target of at least five rats for each time point analysed. C, control (normal) liver; PL; preneoplastic liver, 12, early nodules; N, nodules; H, HCC. (B) Representative immunoprecipitation analysis of FOXM1 and its upstream activators (phosphorylated extracellular signal-regulated kinase (pERK1/2) and glioblastoma-associated oncogene 1 (GLI1)) and downstream effectors (CYCLIN B1, CDC2, AURORA KINASE A (AURKA) and NIMA-RELATED KINASE 2 (NEK2)). Protein lysates were immunoprecipitated with specific antibodies and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The CYCLIN B1–CDC2 complexes were determined through immunoprecipitation (IP) with the anti-CYCLIN B1 antibody and probing the membranes with the anti-CDC2 antibody (immunoblot; IB). (C) Chemiluminescence analysis showing the mean (SD) of at least five rats for each time point investigated. Optical densities of the peaks were normalised to β-actin values and expressed in arbitrary units. Tuckey–Kramer test: A, (•) carcinogen-treated vs control, $p < 0.001$; (*) BN vs F344, $p < 0.001$. C, (•) carcinogen-treated vs control, at least $p < 0.01$; (*) BN vs F344, $p < 0.001$.

USA) antibody was used to immunoprecipitate chromosomal DNA in cross-linked chromatin prepared from exponentially growing HuH6 and HLE cell lines. Immunoprecipitated DNA was analysed by PCR with primers specific for SKP2, CKS1 and CDC2 promoters.[17]

Statistical analysis

Student t test and the Tukey–Kramer test were used to evaluate statistical significance, and Fisher's exact test was used for comparative analysis of survival of HCC patient subgroups. Multiple regression analysis was performed to calculate the correlation coefficient (*R*) using GraphPad InStat 3 (www.

graphpad.com). Values of $p < 0.05$ were considered significant. Results are means (SD).

RESULTS

FOXM1 overexpression in rat and human hepatocarcinogenesis

For the experiments on rat liver tissues, frozen archival material, previously collected,[6][24] was used. At 4 and 6 weeks after initiation, foci of altered hepatocytes occupied 80–97% and 40–50% of the liver in F344 and BN rats, respectively. At 12 weeks, a pool of nodules was used for analysis. They were ∼6-fold smaller in size in BN than in F344 rats. Dysplastic nodules and HCCs (70% moderately differentiated and 30%
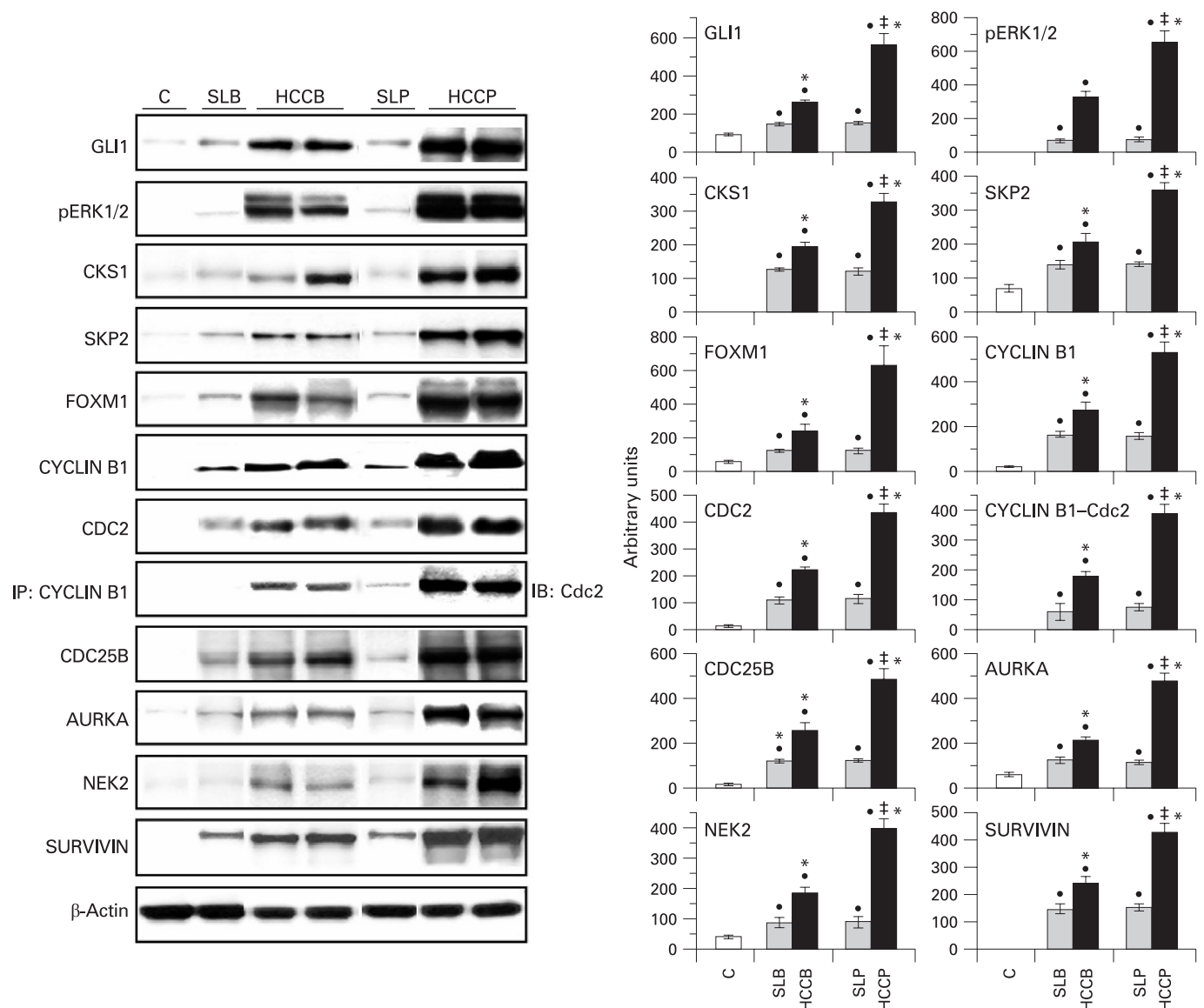
Figure 2 Expression of *FOXM1* (Forkhead box M1B) and related genes in human neoplastic liver lesions. Left panels: representative immunoprecipitation analysis of FOXM1, its upstream activators (phosphorylated extracellular signal-regulated kinase (pERK1/2) and glioblastoma-associated oncogene 1 (GLI1)) and downstream effectors (CYCLIN B1, CDC2, CDC25B, AURORA KINASE A (AURKA), NIMA-RELATED KINASE (NEK2), S-PHASE KINASE-ASSOCIATED PROTEIN 2 (SKP2), SKP2, SURVIVIN and CDC28 PROTEIN KINASE 1b (CKS1)). Protein lysates were immunoprecipitated with specific antibodies and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The CYCLIN B1–CDC2 complexes were determined through immunoprecipitation (IP) with the anti-CYCLIN B1 antibody and probing the membranes with the anti-Cdc2 antibody (immunoblot; IB). Right panels: chemiluminescence analysis showing the mean (SD) of 6 normal livers, 32 HCCBs (hepatocellular carcinomas with better prognosis) and 26 HCCPs (hepatocellular carcinomas with poor prognosis), and corresponding surrounding livers with better or poorer prognosis. Tuckey–Kramer test: (•) HCC and surrounding subtypes vs normal liver, at least $p < 0.001$; (*) HCC subtypes vs corresponding surrounding liver, $p < 0.001$. (‡) Different from HCCB for $p < 0.0001$. SLB and SLP, surrounding liver with better and poorer prognosis, respectively.

poorly differentiated) were present at 32 and 57 weeks, respectively, only in F344 rats. Clear/eosinophilic cell nodules, without atypical features, and HCCs (92% well differentiated) developed in BN rats at 32 and 60 weeks, respectively.

FOXM1 mRNA levels were comparatively evaluated by QRT-PCR in preneoplastic and neoplastic liver lesions from F344 and BN rats. No differences were detected when comparing the expression of *FOXM1* in normal livers and in livers at 4 and 6 weeks after initiation from F344 and BN rats (fig 1A). *FOXM1* upregulation occurred as early as 12 weeks after initiation in F344 lesions and progressively increased in dysplastic nodules and HCCs (fig 1A). In contrast, *FOXM1* upregulation occurred only in nodules and HCCs from resistant BN rats when

compared with control liver values, and always to a lower extent than corresponding lesions in F344 rats (fig 1A). Equivalent results were observed when assessing the expression of FOXM1 and its targets at the protein level. Indeed, FOXM1 and its targets (Aurka, Cdc2, cyclin B1 and Nek2)[11–13 17 27–30] and Cdc2–cyclin B1 complexes were significantly higher at all time points in F344 than BN rat lesions (fig 1B,C).

The levels of FOXM1 and AURKA, NEK2, SURVIVIN, CDC2, CYCLIN B1, CDC25B, SKP2 and CKS1[11–13 17 27–30] downstream targets were assessed at the protein level in human normal livers, HCCs and the respective non-neoplastic surrounding livers (fig 2). A progressive upregulation of FOXM1 and its targets occurred in non-tumorous tissues and HCCs when compared with normal
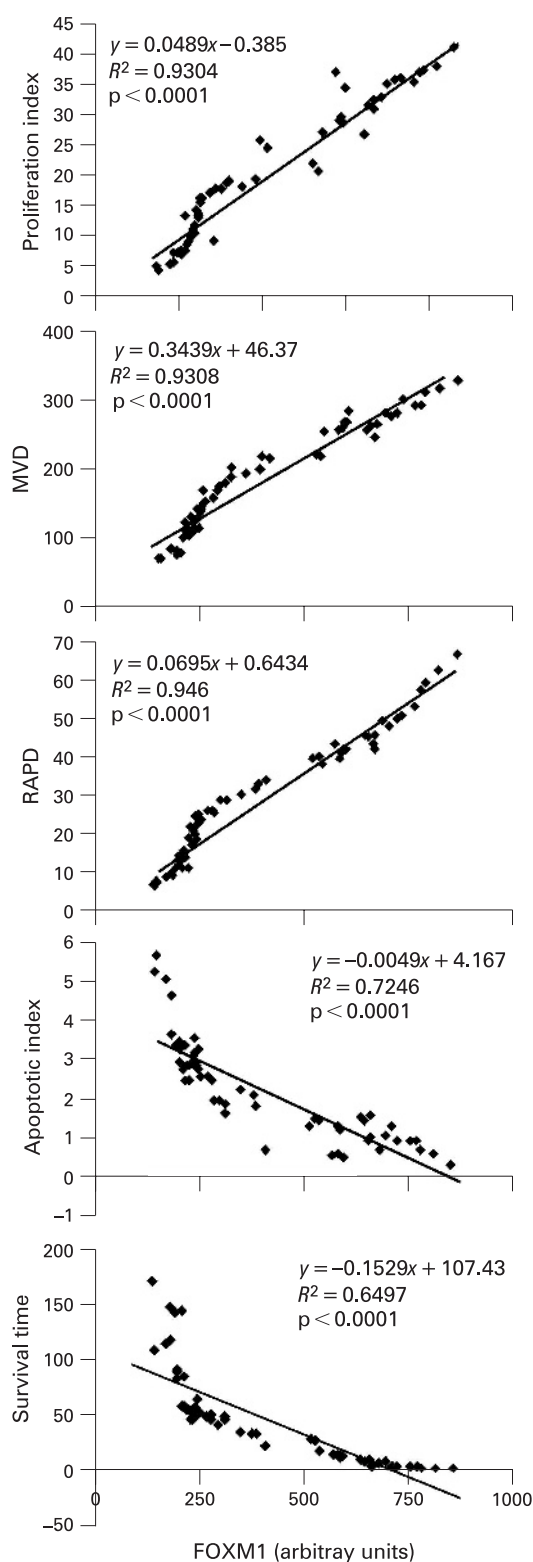
Figure 3 Relationships between FOXM1 (Forkhead box M1B) levels and proliferation index, microvessel density (MVD), random amplified polymorphic DNA (RAPD), apoptotic index (percentage of apoptotic bodies) and length of survival (months after partial liver resection) of human hepatocellular carcinomas (HCCs).

livers, with the highest levels being detected in HCCP tumours. Accordingly, a gradual rise in CDC2–CYCLIN B1 complexes was observed from non-neoplastic liver tissues to tumours, reaching the highest values in HCCP tumours.

These data indicate that activation of the FOXM1 axis is associated with susceptibility to hepatocarcinogenesis in rats and clinical outcome in human HCC.

Correlation of FOXM1 levels with clinicopathological parameters in human HCC

The proliferation index, microvessel density and genomic instability (RAPD) values were 2.5- to 3-fold higher in HCCP than HCCB (Supplementary table 1) and correlated with FOXM1 levels (fig 3). In contrast, an inverse correlation of FOXM1 expression with apoptosis and patients' length of survival was found (fig 3). No significant correlation between FOXM1 and other clinicopathological parameters, including aetiology, sex, age, presence of cirrhosis, α-fetoprotein, tumour size and grading was observed.

FOXM1 activation is mediated by ERK and GLI1 in HCC cell lines

Previous reports showed FOXM1 upregulation following either ERK or GLI1 induction.[31] [32] To identify the upstream inducer(s) of FOXM1 in HCC, we first assessed the levels of activated ERK and GLI1 in both rat and human lesions. Phosphorylated ERK (pERK1/2) and GLI1 protein levels rose at 12 weeks after initiation and increased in neoplastic nodules and HCCs from F344 rats (fig 1B). In BN rats, pERK1/2 and GLI1 upregulation was limited to nodules and HCCs, but to a lower extent than in corresponding F344 lesions. Furthermore, pERK1/2 and GLI1 expression was higher in human HCCs than in normal and non-neoplastic surrounding livers, and most pronounced in HCCP (fig 2). Next, we assessed the impact of suppressing ERK proteins and GLI1 on FOXM1 expression in human HuH6 and HLE HCC cell lines, displaying elevated *FOXM1* mRNA levels (not shown). Silencing of either *ERK2* or *GLI1* via siRNA led to moderate decreases in GLI1 and ERK2 protein levels, respectively, and to stronger decreases in FOXM1 levels. Noticeably, the combined suppression of ERK2 and GLI1 resulted in almost complete inhibition of FOXM1 (fig 4A, Supplementary fig S1a). Conversely, upregulation by transient transfection with *ERK2* or *GLI1* resulted in increases in GLI1 and pERK1/2, respectively, and in an elevated FOXM1 level which further increased in doubly transfected cells (fig 4B, Supplementary fig S1b). These findings imply an additive activity of ERK and GLI1 in promoting FOXM1 upregulation.

FOXM1 sustains ERK activity via degradation of DUSP1

Recent results showed that human kidney embryonic cells[33] and rat and human HCCs[8] [10] maintain elevated levels of ERK via downregulation of its specific inhibitor, DUSP1. In these cells, DUSP1 downregulation was achieved by its proteolysis mediated by cooperation bertween ERK, SKP2 and CKS1.[10] Previous[17] and present data suggest that CKS1 and SKP2 are FOXM1 targets. In accordance with this hypothesis, we detected the functional interaction of FOXM1 with SKP2 and CSK1 proteins in HuH6 and HLE cells by ChIP analysis (fig 5). Thus, we examined the role of FOXM1 in DUSP1 suppression. Strikingly, inhibition of FOXM1 expression by siRNA in HuH6 and HLE cell lines led to marked CKS1 and SKP2 downregulation, with consequent upregulation of DUSP1, a decrease in levels of ubiquitinated DUSP1 and a strong reduction in ERK activity (fig 4C, Supplementary fig S1c). Conversely, over-expression of FOXM1 in SNU-182 HCC cells (exhibiting low FOXM1 levels) triggered DUSP1 downregulation, which was inhibited by siRNA against SKP2 and CKS1 (fig 4D, Supplementary fig S1d). These observations assign a role to FOXM1 in sustaining ERK activation via downregulation of the ERK inhibitor DUSP1.
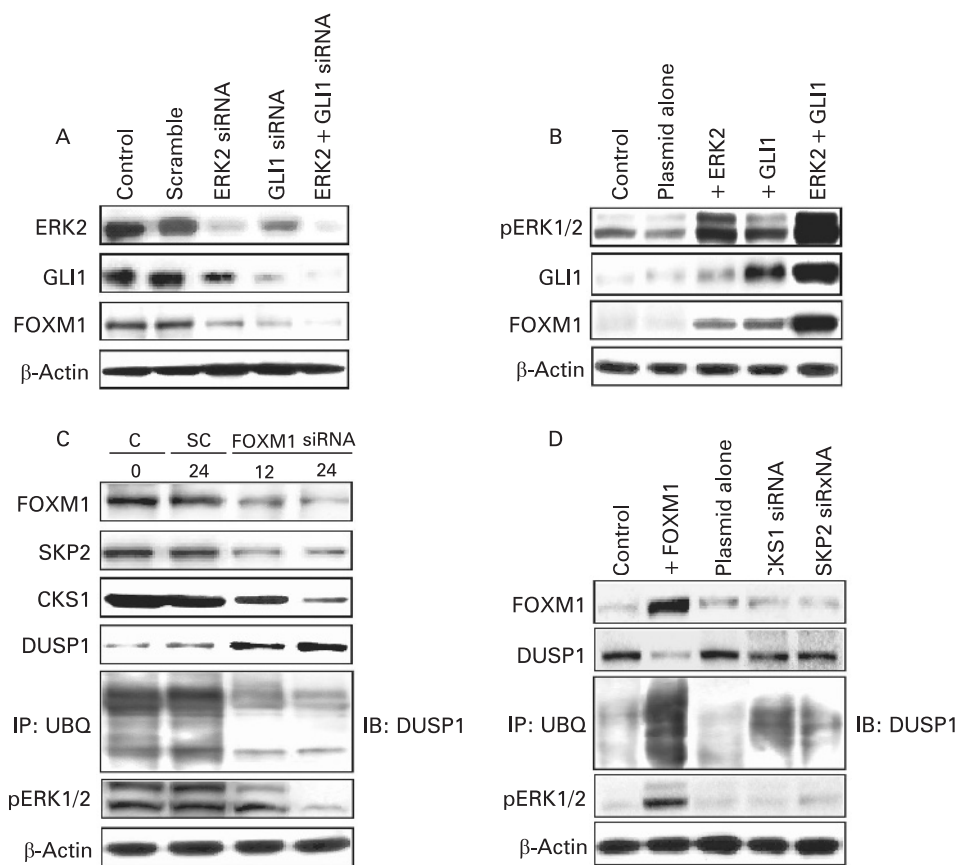
Figure 4 Representative immunoprecipitation analysis of the effects of the inhibition of FOXM1 (Forkhead box M1B) upstream regulators (extracellular signal-regulated kinase (ERK) and glioblastoma-associated oncogene 1 (GLI1)) and changes in FOXM1 protein level on its downstream effectors S-phase kinase-associated protein 2 (SKP2) and CDC28 protein kinase 1b (CKS1) in human hepatocellular carcinoma (HCC) cell lines. Ubiquitinated dual-specificity phosphatase 1 (DUSP1) was determined by immunoprecipitation (IP) of ubiquitin (UBQ) followed by immunoblotting (IB) with antibodies against DUSP1. (A) The HuH6 cell line was treated for 48 h with small interfering RNA (siRNA) against $ERK2$, $GLI1$, or $ERK2+GLI1$. Equivalent results were obtained using the HLE cell line (data not shown). (B) SNU-182 cells were transfected with $ERK2$ (wild-type) in a pUSEamp plasmid (Millipore), and $FOXM1$ and $GLI1$ (wild-type) cDNA in a pCMV6-XL vector. (C) The HuH6 cell line was treated for 12 and 24 h with siRNA against FOXM1. Equivalent results were obtained using the HLE cell line (data not shown). (D) The SNU-182 cell line, transiently transfected with FOXM1, was treated for 36 h with siRNA against CSK1 or SKP2. Controls (C) received solvent alone or scramble oligonucleotides.

FOXM1 promotes HCC cell proliferation, survival and angiogenesis

The role of FOXM1 in HCC cells was investigated by assessing the consequence of FOXM1 inactivation in HuH6 and HLE cells by siRNA and of FOXM1 overexpression on SNU-182 cells, respectively (fig 6). FOXM1 suppression markedly reduced proliferation, and EPO and VEGFα secretion, and induced apoptosis. In sharp contrast, FOXM1 overexpression led to an increase in cell proliferation, and in EPO and VEGFα secretion, (potentially) contributing to neovascularisation,[34 35] and a decline in apoptosis. The growth suppression by siRNA was studied further in HuH6 cells by flow cytometric analysis, which revealed a cell cycle arrest at G_0–G_1 and G_2–M phases.
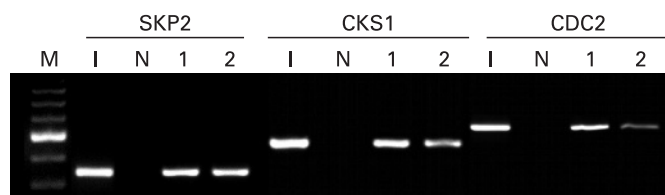

Figure 5 Functional interaction of FOXM1 (Forkhead box M1B) with $SKP2$ (S-phase kinase-associated protein 2), $CKS1$ (CDC28 protein kinase 1b) and $CDC2$ genes in human HuH6 and HLE hepatocellular carcinoma (HCC) cell lines as detected by chromatin immunoprecipitation analysis. M, DNA marker; I, input (aliquot of chromatin prior to immunoprecipitation); N, negative control (absence of DNA in the PCR); 1, HuH6; 2, HLE. Cross-linked chromatin was immunoprecipitated with the anti-FOXM1 antibody. Chromatic immunoprecipitation of the $CDC2$ gene by FOXM1 was also performed as a positive control.

DISCUSSION

Recent work indicates that a peculiar feature of hepatocarcinogenesis is that changes of signal transduction in autonomously growing preneoplastic and neoplastic cells are under control of the genes responsible for susceptibility to HCC development.[4] Deregulation of G_1 and S phases of the cell cycle, implying fast G_1–S transition and elevated DNA synthesis, in lesions of the susceptible F344 rat, is considerably less remarkable in lesions of the resistant BN rat.[4 6] Here, we show an earlier and more pronounced FOXM1 induction associated with a faster growth of preneoplastic and neoplastic liver lesions in susceptible F344 than in resistant BN rats. FOXM1 upregulation was followed by a very prominent rise in FOXM1 targets in F344 preneoplastic and neoplastic livers, including some proteins implicated in G_2–M transition, such as Cdc2, cyclin B1, AurkA and Nek2.[11–17 27–30]
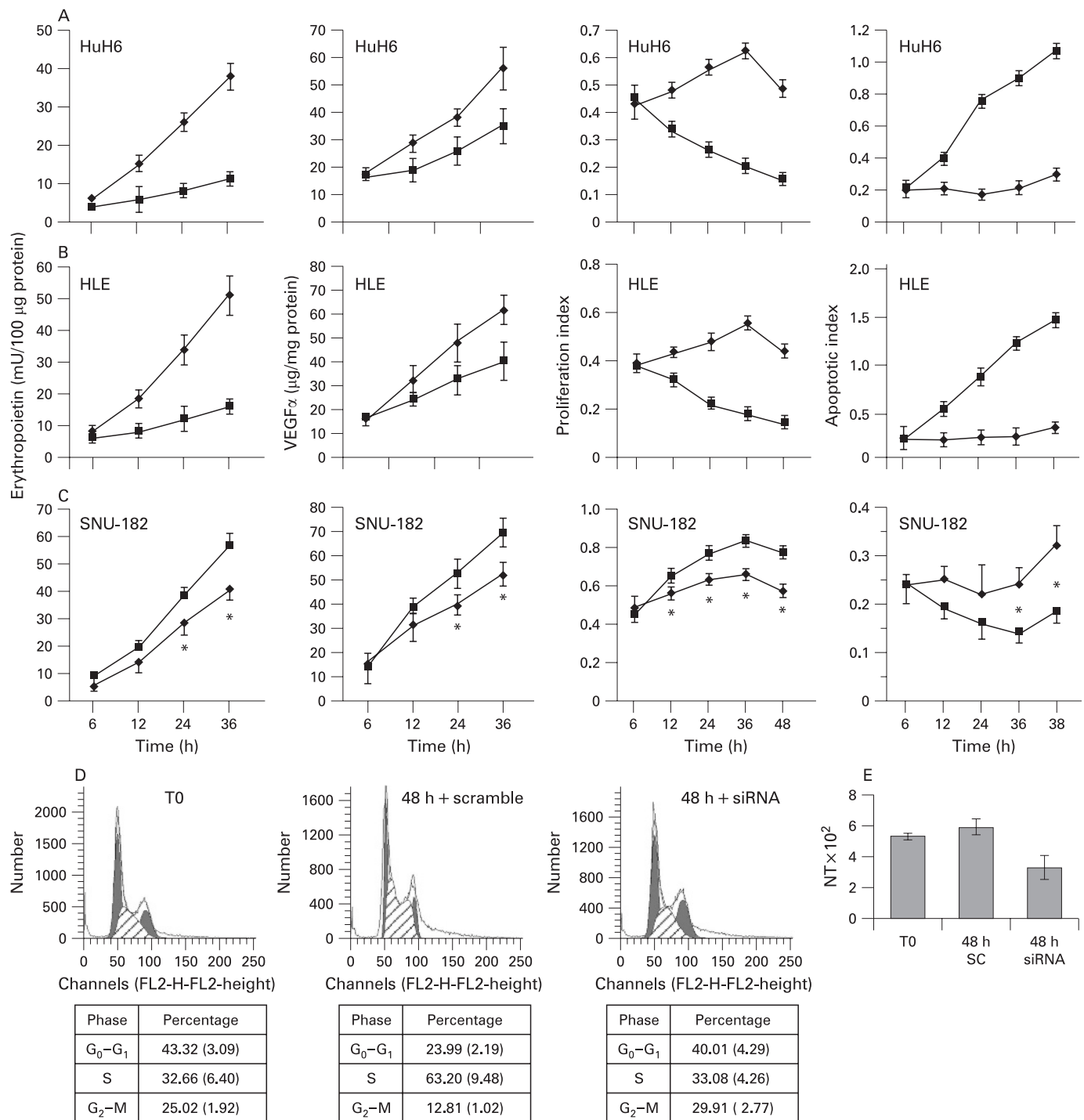
Figure 6 Effect of the modulation of *FOXM1* (Forkhead box M1B) expression on proliferation and apoptosis of human hepatocellular carcinoma (HCC) cell lines. (A) and (B) Effect of inhibition of *FOXM1* by small interfering RNA (siRNA) on erythropoietin and vascular endothelial growth factor α (VEGFα) production, proliferation, and apoptosis in HuH6 and HLE cells, respectively. (C) Effect of FOXM1 overexpression on erythropoietin and VEGFα production, proliferation and apoptosis in SNU-182 cells. The cells were transiently transfected with *FOXM1* cDNA in a pCMV6-XL vector. Results in A–C are means (SD) of five experiments. siRNA-treated or transfected cells and controls are shown by squares and rhombuses, respectively. Controls received scramble oligonucleotides (SC) or plasmid alone. (D) Representative flow cytometric analysis and average quantitative data (SD) of three independent experiments with HuH6 cells, transfected with anti-FOXM1 siRNA. The analyses were performed at the end of culture synchronisation, in low serum medium (T_0), and after 48 h incubation with SC or siRNA. Quantitative evaluations were made by ModFit LT (Verity Software House, Topsham, Maine, USA). (E) *FOXM1* mRNA levels determined by quantitative reverse transcription-PCR. N Target $= 2^{-\Delta Ct}$; $\Delta Ct = Ct$ RNR18–Ct target gene. Data are means (SD) of N target of three experiments. Tuckey–Kramer test, treated vs control: (A) and (B), $p < 0.001$ at all time points after 6; (C), $p < 0.001$ at the asterisked time points. "t" test: (D) siRNA vs SC, $p = 0.0045$, $p = 0.0075$ and $p < 0.001$ for G_0–G_1, S and G_2–M, respectively. (E) siRNA vs SC, $p = 0.0045$.

Accordingly, restriction of FOXM1 expression by siRNA, in the HuH6 cell line, led to G_2–M phase arrest. FOXM1 inhibition was also associated with G_0–G_1 arrest which could depend on modulation of the activity of FOXM1 targets, including pERK1/2 downregulation,[36] and possible activation of G_1 inhibitors such as p21[WAF1] and p27[KIP1].[14–17] Thus, the present investigation shows FOXM1 regulation by genes controlling the susceptibility to HCC, and underlines the role of uncontrolled progression through the cell cycle as an effector mechanism of these genes, determining the susceptible phenotype.

In human samples, FOXM1 was ubiquitously and progressively induced from non-tumorous surrounding liver to HCC, with the highest increase in HCCP, substantiating the role of FOXM1 in both HCC development and progression, in accordance with mouse[18] and rat (present work) hepatocarcinogenesis. FOXM1 levels directly correlated with genomic instability, the proliferation index and tumour microvessel density, and inversely with the apoptotic index and survival, indicating that FOXM1 contributes to hepatocarcinogenesis via multiple mechanisms. Indeed, FOXM1 upregulation induced overexpression of genes promoting cell cycle progression (*AURKA, CDC2, CYCLIN B1, NEK2* and *CDC25B*), generation of genomic instability (*NEK2* and *CDC25B*), suppressors of cell cycle inhibitors (*SKP2* and *CKS1*) and apoptosis inhibitors (*SURVIVIN*)[11–17 27–30] (Supplementary fig S2). Importantly, the strong correlation between FOXM1 levels and both genomic instability rate and adverse outcome in HCC agrees with the existence of a molecular signature, including *FOXM1* overexpression, which is significantly associated with the degree of genomic instability and predicts survival of patients with multiple tumours.[37] Furthermore, 9 of 70 (12.9%) genes representing the signature of genomic instability and short survival are direct FOXM1 targets (*CYCLIN B1* and *B2, CDC2, NEK2, KIF20A, TOP2A, CDC25B, AURORA KINASE A* and *AURORA KINASE B*).[11–17 27–30] These data, together with the results from the comparative analysis of preneoplastic and neoplastic lesions of rats with different genetic predisposition to hepatocarcinogenesis and human HCC with different prognosis, suggest a potential prognostic role for FOXM1 signalling in numerous neoplasms. In addition, induction of EPO and VEGFα expression substantiates the role of FOXM1 in HCC neoangiogenesis. Signalling via EPO and the EPO receptor is required for angiogenesis in numerous non-haematopoietic tissues[34 35] and in cancer, including human pancreatic cancer,[16] and mouse and human HCC cells.[38 39]

A link between fast growth and signalling deregulation characterises human HCCP, whereas the behaviour of HCCBs is more similar to that of resistant rat lesions[4 6 22] (and present work). This does not necessarily imply a genetic regulation of signalling pathways in humans like that found in rodents, in which polygenic inheritance with several low penetrance genes and a main gene regulates the genetic predisposition to HCC.[5] Even if according to epidemiological studies a genetic model, similar to that of rodents, can influence human hepatocarcinogenesis,[5] further studies are needed to clarify the influence of susceptibility genes on signalling pathways supporting tumour growth and progression in humans.

Furthermore, we demonstrated the importance of FOXM1 in sustaining ERK activity by inducing CKS1 and SKP2 expression. SKP2/CSK1 ligase, which degrades DUSP1, a major ERK inhibitor, contributes to sustained ERK overactivity in HCC.[10] On the other hand, sustained ERK2 activation triggers DUSP1 degradation via phosphorylation of its Ser296 residue, followed by ubiquitination and proteasomal degradation (Supplementary fig S2).[10] Therefore, FOXM1 is involved in a positive feedback loop, reinforcing the ERK cascade by its ability to inhibit DUSP1.

The possible upstream inducers of FOXM1 were also investigated. FOXM1 is a direct transcriptional target of GLI1.[40] GLI family proteins, including GLI1, 2 and 3, are the terminal effectors of Hedgehog signalling.[41 42] The interaction of Sonic hedgehog (SHH) with its plasma membrane receptor PTCH1, releases PTCH-induced inhibition of the membrane protein Smoothened (SMO). This results in activation and nuclear translocation of GLI proteins, where they activate target gene transcription.[43] GLI2 is overexpressed in some HCC cell lines, and its inhibition by antisense oligonucleotides inhibits cell proliferation.[44] GLI1 overexpression occurs in a lower number of HCC cell lines than GLI2 and its inhibition causes a lower decrease in growth rate.[44] We did not evaluate the effect of GLI2 on FOXM1 activity. It must be considered that the effect of GLI proteins on cell proliferation may reflect changes in different genes and signalling pathways. Our data suggest that FOXM1 upregulation results from combined ERK and GLI1 activity in HCC. The elevated levels of both ERK proteins and GLI1 in F344 rat liver lesions and HCCP might therefore explain the highest levels of FOXM1 in these lesions. Our results suggest a reciprocal activation of *ERK2* and *GLI1*. *GLI1* has a MEK-1-responsive N-terminal domain, and a recent report indicates that the activation of the ERK pathway by basic fibroblast growth factor stimulates *GLI1* activity through this domain.[45] The mechanism underlying ERK stimulation by the Hedgehog pathway is unclear. Indirect ERK activation through GLI-mediated induction of platelet-derived growth factor (PDGF) receptor α has been postulated.[46] Present knowledge suggests a complex cross-talk between Hedgehog and MEK/ERK signalling whose role in hepatocarcinogenesis requires further investigation. Interestingly, a recent report implies the combined overexpression of HSP90 and CDC37 proto-oncogenes in sustaining elevated Fused Homolog expression.[47] Accordingly, our preliminary data indicate the upregulation of the Fused Homolog gene in human HCC (results not shown), and a previous report showed a strong induction of HSP90 and CDC37 in F344 rat liver lesions and human HCCP.[24] Thus, a role for combined activity of HSP90 and CDC37 in the highest activation of GLI1 observed in F344 neoplastic lesions and human HCCPs might be hypothesised.[24] Overall it seems that FOXM1 acts as a pleiotropic regulator of human hepatocarcinogenesis, playing multiple roles on preneoplastic and neoplastic hepatocytes.

Overall, our results indicate for the first time a genetic control of FOXM1 signalling deregulation during hepatocarcinogenesis and its role in both HCC development and prognosis. Furthermore, we show the involvement of FOXM1 in a positive feedback loop in which the activating interaction of FOXM1 with the SKP2/CSK1 ligase sustains ERK and FOXM1 overactivity. This mechanism may have a central role in the pathogenesis of fast growing HCCs. The association of the block of FOXM1 signalling by specific siRNA with a consistent decrease in HCC cell growth and EPO production, and an increase in apoptosis in vitro, suggests that FOXM1 could represent a therapeutic target that, in association with other targets, may contribute to create networked biological treatments.[48]

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