Promoter occupancy of MLL1 histone methyltransferase seems to specify the proliferative and apoptotic functions of E2F1 in a tumour microenvironment

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Summary

The E2F family of transcription factors are considered versatile modulators, poised at biological crossroads to execute diverse cellular functions. Despite extensive studies on E2F, the molecular mechanisms that control specific biological functions of the E2F1 transcription factor are still not fully understood. Here we have addressed the molecular underpinnings of paradoxical functions of E2F1 in a tumour microenvironment using the 'X15-*myc*' oncomouse model of hepatocellular carcinoma. We observed that the HBx oncoprotein of hepatitis B virus regulates E2F1 functions by interfering with its binding to Skp2 E3 ubiquitin ligase. The HBx–Skp2 interaction led to the accumulation of transcriptionally active E2F1 and histone methyltransferase mixed lineage leukemia 1 (MLL1) protein. During early stages of hepatocarcinogenesis, the increased E2F1 activity promoted cellular proliferation by stimulating the genes involved in cell cycle control and replication. However, during the late stages, E2F1 triggered replication-stress-induced DNA damage and sensitized cells to apoptotic death in a p53-independent manner. Interestingly, the different promoter occupancy of MLL1 during the early and late stages of tumour development seemed to specify the proliferative and apoptotic functions of E2F1, through its dynamic interaction with the co-activator CBP or co-repressor Brg1. Thus, the temporally regulated promoter occupancy of histone methyltransferase could be a regulatory mechanism associated with the diverse cellular functions of the E2F family of transcription factors.

Key words: Apoptosis, DNA damage, E2F1, HBx, MLL1, Replication stress, X15-*myc* mice

Introduction

In metazoans, cell proliferation is permitted until they sense genotoxic stress. Such genotoxic insults either stimulate cell repair processes or induce apoptotic cell death. Thus, the life and death decisions are context dependent and are finely executed by the cell surveillance mechanisms (Zhivotovsky and Kroemer, 2004). While mitogenic stimuli drive cells to proliferate, their absence might trigger a stress response leading to apoptotic death (Evan and Vousden, 2001). The E2 promoter binding factor (E2F) family of transcription factors functions as an important mediator of cell proliferation as well as DNA-damage-induced apoptotic response (Polager and Ginsberg, 2008). The E2F factors consist of two major functional groups, the transcriptional activators (E2F1–E2F3) and transcriptional repressors (E2F4–E2F7), both known to act by binding to specific DNA response elements of the target promoters and regulating gene expression (Attwooll et al., 2004). This simplistic view of the E2F family has been challenged because of the observation that E2Fs also exhibit tumour suppressor activity in addition to their canonical role as oncogenes (Johnson and Degregori, 2006). Such dual roles of E2F may be dependent on cellular context, in which pathways controlling cell cycle progression and apoptosis are intimately linked (Johnson, 2000). Importantly, E2F1 gene amplification and its aberrant expression have been reported in many human cancers (Rogoff and Kowalik, 2004). As E2F1 discharges multiple cellular functions including maintenance of genomic integrity, its levels are under the tight control of transcriptional and post-translational regulatory mechanisms (Wu et al., 2009).

Thus far, the paradoxical functions of E2F1 have received adequate attention but the molecular interplay that specifies the E2F1 paradox under different biological contexts is not well understood (Polager and Ginsberg, 2009). The C-terminal region of E2F1 carries a site for ubiquitylation that remains masked because of its interaction with retinoblastoma-associated protein (Rb) (Hofmann et al., 1996). Multiple E3 ubiquitin ligases have been implicated in the regulation of E2F1. Prominent among these being the anaphase-promoting complex/cyclosome^{Cdc20} (APC/C^{Cdc20}) during pro-metaphase (Peart et al., 2010), APC/C^{Cdh1} during early G1 phase (Budhavarapu et al., 2012) and SCF^{Skp2} during S–G2 phases (Marti et al., 1999). Besides, E2F1 may be degraded by nucleolar ubiquitin proteasome through the p19(ARF) pathway (Martelli et al., 2001). Thus, the ubiquitylation of E2F1 might have a role in fine-tuning of the Rb–E2F axis.

The Rb–E2F axis is a major regulatory node, which is often found to be deregulated in most human cancers including

hepatocellular carcinoma (HCC) (Chen et al., 2009). However, there is no evidence to link the ubiquitylation status of E2F1 with its deregulated activity in the tumour microenvironment. The deregulation of E2F1 activity has been reported in an oncogene-induced apoptosis model that implicates the Rb–E2F axis (Nahle et al., 2002). The Rb–E2F1 interaction has a different connotation as a repressor E2F1 complex and includes histone deacetylases (HDACs), the chromatin-remodelling complex SWI/SNF (having Brg1/Brm as an ATPase subunit) and the polycomb group of proteins. In contrast, the active or free E2F1 recruits histone acetyl transferases (HATs) and H3K4 histone methyl transferases (HMTs) to form co-activator complexes to target gene activation (Wilson, 2007). The histone methyltransferase mixed lineage leukemia 1 (MLL1) protein is also known to associate with E2F-responsive promoters, suggesting its possible role in cell cycle progression (Tyagi et al., 2007). Interestingly, MLL is also degraded bi-modally similar to E2F1, with the help of SCF^{Skp2} and APC/C^{Cdc20} E3 ligases to ensure the bi-phasic expression of MLL (Liu et al., 2007).

In the present study, we addressed the molecular underpinnings of E2F1 paradox in a hepatic tumour microenvironment of the 'X15-*myc*' oncomouse model as this offers an *in vivo* milieu to monitor tumour development. In this transgenic model, a truncated form of the HBx oncogene (X15) of hepatitis B virus is co-expressed with the *myc* gene in the liver. Previously we reported that 'X15-*myc*' oncomice develop hepatocellular carcinoma (HCC) leading to their death by 20–22 weeks of age (Lakhtakia et al., 2003). Working on this model, we observed that viral HBx competed with E2F1 for its binding to Skp2 ubiquitin E3 ligase, leading to the accumulation of transcriptionally active E2F1. The active E2F1 stimulated cell cycle and replication-associated genes in the early stage tumours whereas in the late stage tumours, it triggered replicational stress-induced DNA damage and sensitized cells for apoptosis. Importantly, the promoter occupancy of H3K4 histone methyl transferase MLL1 seemed to specify the proliferative and apoptotic functions of E2F1. Furthermore, the formation of mutually exclusive E2F1 complexes either with co-activator CBP or co-repressor Brg1 seemed to regulate the promoter-specific sequestration of MLL1.

Results

HBx confers intracellular stability to E2F1 protein through a post-translational mechanism

Because E2F1 levels are generally de-regulated in many human cancers, we measured its levels in the liver of the X15-*myc* oncomouse. Western blot analysis of the tissue lysates suggested significant accumulation of E2F1, compared with control animals, as early as 1 month after birth (Fig. 1A). Interestingly, similar results were also observed in HepG2.2.15 cells that carry the stably integrated HBV genome in HepG2 background (Fig. 1B). Furthermore, our inhibitor studies in the presence of both α-amanitin and cycloheximide (CHX) suggested that unlike HepG2 cells, there was an accumulation of E2F1 in HepG2.2.15 cells despite the inhibition of nascent mRNA and protein synthesis (Fig. 1C,D). As the half-life of E2F1 mRNA is 6 hours (Saunders et al., 1998) and the protein 70 minutes (Campanero and Flemington, 1997), the accumulation of E2F1 in HepG2.2.15 cells could be possibly through post-translational mechanisms regulated by HBx. Thus, these results suggested a novel role of HBx in E2F1 stabilization, independent of its well-known function as a transcriptional regulator of E2F1 (Jung et al., 2007).

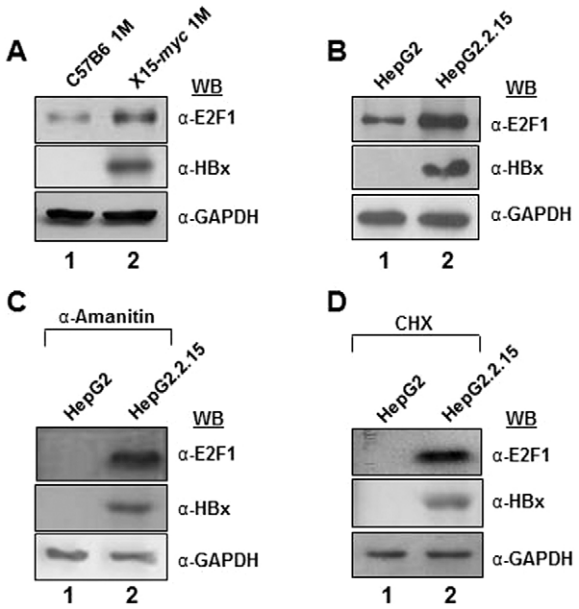

Fig. 1. HBx-mediated regulation of E2F1 protein. (A,B) Western blots of liver tissue lysates from 1-month-old C57B6 control mice and X15-*myc* transgenic mice (A) and lysates of HepG2 and HepG2.2.15 cells (B) using anti- E2F1, -HBx and -GAPDH. (C,D) HepG2 and HepG2.2.15 cells were either treated with α-amanitin for 24 hours (C) or treated with cycloheximide (CHX) for 4 hours (D) and the cell lysates were subjected to western blotting for E2F1 and HBx. GAPDH was used as loading control.

HBx interaction with Skp2 prevents ubiquitylation and degradation of E2F1

To decipher the post-translational mechanism involved in E2F1 stabilization, we performed pulse-chase analysis of E2F1 in HepG2 and HepG2.2.15 cells. Consistent with the 70 minute half-life of E2F1, we observed a gradual decline in the E2F1 levels in HepG2 cells. In contrast, HepG2.2.15 cells showed sustained levels of E2F1 until 3 hours (Fig. 2A). Analysis of the ubiquitylation status of E2F1 in HepG2 cells revealed more polyubiquitylated forms of E2F1 in the absence of HBx, as in cells treated with MG132 (a potent inhibitor of 26S proteasome activity; Fig. 2B, lanes 1, 2). However, upon ectopic expression of HBx, a decrease in the polyubiquitylated forms of E2F1 was observed (lane 3). The residual ubiquitylation found under these conditions was abolished upon overexpression of DP1 (lane 4). Thus, HBx seems to mediate E2F1 stability by blocking its ubiquitylation by Skp2 and its subsequent proteasomal degradation.

Earlier we showed that HBx can confer intracellular stability to Myc by preventing its ubiquitylation by the E3 ubiquitin ligase Skp2 (Kalra and Kumar, 2006). To explore whether a similar mechanism was responsible for E2F1 stability, we analysed the HBx–Skp2–E2F1 interactions in the HBx microenvironment. As shown in Fig. 2C, Skp2 interacted with E2F1 in HepG2 cells but not in HepG2.2.15 cells. The Skp2–E2F1 interaction in HepG2.2.15 cells was, however, restored by silencing HBx expression using specific shRNA, suggesting a competitive inhibition of E2F1 binding to Skp2. As expected, a strong Skp2–HBx interaction was observed in HepG2.2.15 cells. Dissociation of Skp2 from other components of the SCF complex such as Skp1 and CUL1 could also be seen (Fig. 2C, lane 2). Interestingly, a similar pattern of HBx–Skp2 interaction

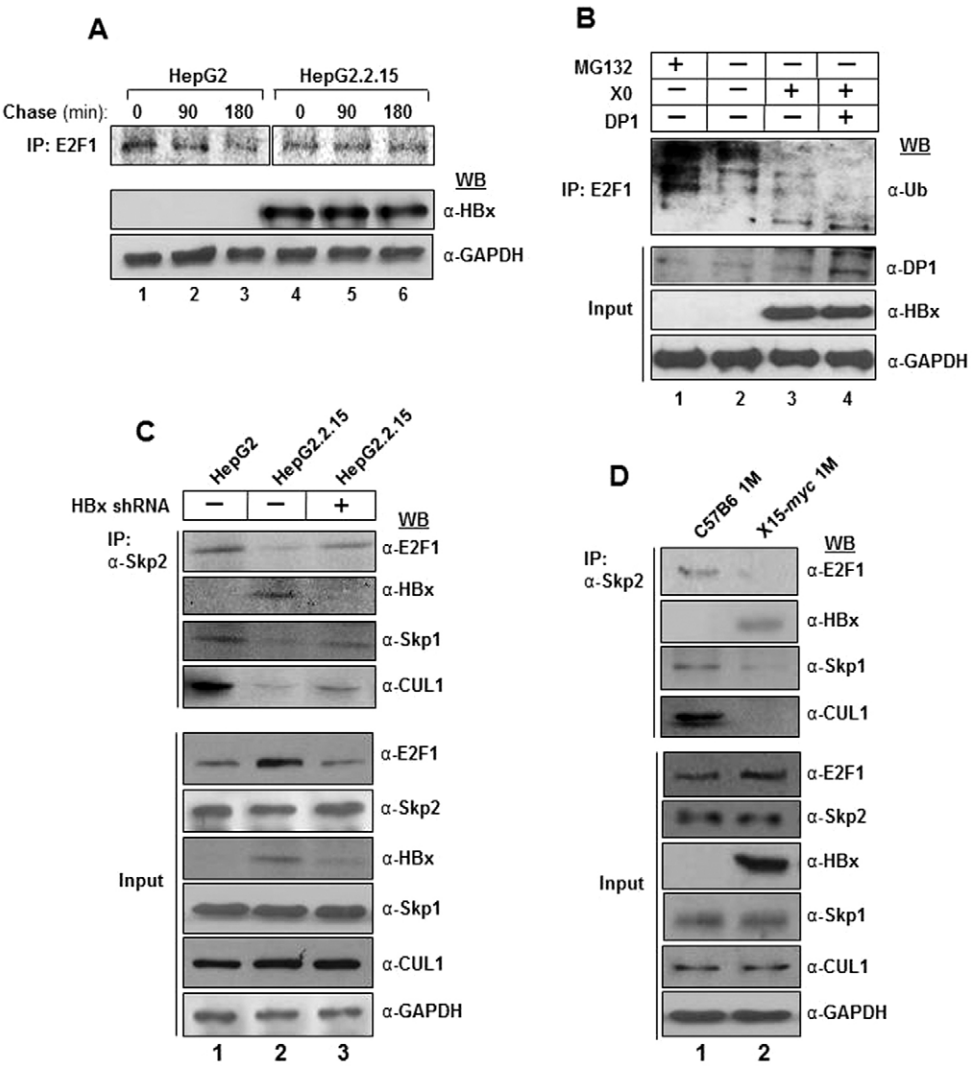

Fig. 2. HBx–Skp2 interaction in the deregulation of E2F1 ubiquitylation and degradation. (A) HepG2 and HepG2.2.15 cells were pulse labelled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 60 minutes and chased for the indicated time periods. The cell lysates were immunoprecipitated with anti-E2F1, resolved by 10% SDS-PAGE and autoradiographed. Expression of HBx was also confirmed in these samples. (B) Immunoprecipitation (IP) of E2F1 from lysates of HepG2 cells given different treatments: no treatment (lane 2), cells treated with MG132 (lane 1), cells transfected with HBx expression vector (X0) alone (lane 3) or X0 co-transfected with DP1 expression plasmid (lane 4). The IP samples were immunoblotted for ubiquitin (Ub). One-tenth of the cell lysate was also used to confirm the expression of DP1 and HBx. (C,D) Immunoprecipitation with anti-Skp2 followed by immunoblotting of E2F1, HBx, Skp1 and CUL1 were performed either on the cell lysates of HepG2-, HepG2.2.15- and HBx-shRNA-transfected HepG2.2.15 cells (C) or tissue lysates of 1-month-old C57B6 control and X15-*myc* transgenic mice (D). One-tenth of the cell lysate was used to confirm the expression of HBx and probed for total levels of Skp2, E2F1, Skp1 and CUL1. In all these experiments, GAPDH levels were used as loading control.

and disruption of the SCF complex were also observed in the tissue lysates of X15-*myc* transgenic mice (Fig. 2D). Together these results suggested that HBx can stabilize E2F1 by preventing its interaction with Skp2 and thereby blocking its subsequent ubiquitylation and degradation.

The HBx-stabilized E2F1 is transcriptionally active

Because HBx appeared to block the ubiquitylation of E2F1 (Fig. 2B), next we probed the binding status of Rb to E2F1 because an overlapping region of E2F1 [amino acids (aa) 409–426] maps to its ubiquitylation as well as Rb binding functions (Hofmann et al., 1996). We observed that decrease in E2F1 ubiquitylation in the HBx microenvironment correlated with decreased total Rb–E2F1 interaction perhaps because of the hyperphosphorylation of Rb (supplementary material Fig. S1). This increase in the levels of free E2F1 suggested the accumulation of the transcriptionally active form of E2F1. Therefore, we analysed the downstream targets of E2F1 in X15-*myc* mice of different ages. We observed a substantial upregulation of cell cycle and replication control proteins such as cyclin E, Cdk2, ORC1, Cdc6, MCM2-7, RPA and PCNA only in the young (3 months) mice but not in 6-month-old mice (Fig. 3A). The E2F1 specificity in the upregulation of its target proteins in the HBx microenvironment was further confirmed by RNA interference studies. As evident from Fig. 3B, increase in the expression of E2F1-specific target genes seen in the presence of HBx was attenuated by E2F1 siRNA. Note, however, that cyclin E, Cdk2 and Orc1 levels showed only a partial inhibition, which might be due to their regulation by alternative mechanisms. Interestingly, analysis of the stability of origin recognition complex 1 (Orc1) protein revealed a much longer half-life (>4 hours) in HepG2.2.15 cells as compared to ~50 minutes in HepG2 cells, leading to its accumulation in the HBx microenvironment (supplementary material Fig. S2). As Orc1 is a substrate of Skp2 for ubiquitylation and degradation, just as E2F1 (Méndez et al., 2002), these results suggested that HBx can increase Orc1 levels through Skp2 inhibition, unlike other replication control proteins that are transcriptionally upregulated by E2F1.

E2F1-mediated unscheduled replication activity results in replicational-stress-induced DNA damage and apoptosis

Because E2F1 was found to upregulate many replication control proteins in the HBx microenvironment, we wondered whether this has any direct effect on the replication activity of genomic DNA. To address this, we measured the occupancy of replication initiation proteins by a chromatin immunoprecipitation (ChIP) assay followed by qPCR of human lamin B2 origin. Compared to

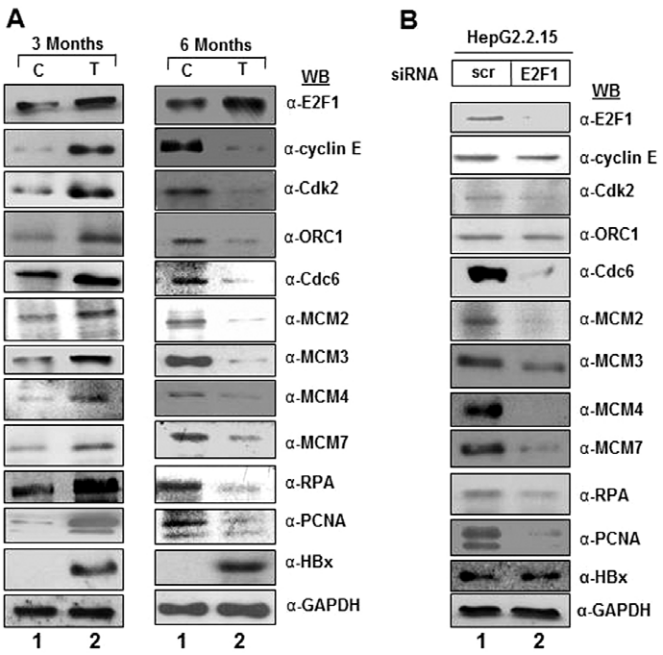

Fig. 3. Regulation of E2F1 target genes involved in the control of cell cycle and DNA replication in the presence of HBx. Western blot analysis of proteins in tissue lysates of 3- and 6-month-old C57B6 control and X15-*myc* transgenic mice (A), or in lysates of HepG2.2.15 cells transfected with either scrambled (scr) or E2F1-specific siRNA (B). GAPDH levels were used as loading control.

HepG2 cells, the HBx expressing HepG2.2.15 cells showed a two- to fourfold increase in the enrichment of Orc1, Cdc6, MCM2, MCM4 and MCM7 proteins that was reversed in the presence of dominant-negative E2F1 (E2F1 DN; Fig. 4A). No change in Cdc45 occupancy was observed because it is a non-target of both E2F1 and HBx. However, considering that Cdc45 is one of the rate-limiting factors for pre-initiation complex formation (Wu and Nurse, 2009), the loss of balance between Cdc45 with other pre-replication complex factors could lead to replication-stress-induced DNA damage (Burhans and Weinberger, 2007). To address this, we measured the levels of γ-H2A.X which is a marker of aberrant DNA structures and damage (Rogakou et al., 1998). We found increased levels of phosphorylated H2A.X (γ-H2A.X) in the HBx microenvironment that was inhibited in the presence of E2F1 DN (Fig. 4B).

E2F1 is also known to support apoptotic DNA damage response (DDR) in a p53-dependent or -independent manner (Bates et al., 1998; Moroni et al., 2001), so next we analysed selected E2F1 targets involved in apoptosis. The elevated levels of pro-apoptotic proteins such as BID, initiator caspases (casp-8 and -9), effector caspases (casp-3 and -7) and Apaf-1 (co-activator of casp-9) were observed in HepG2.2.15 cells. As expected, the levels of these apoptotic markers were reversed in the presence of E2F1 siRNA (Fig. 4C), suggesting that E2F1 was competent to execute the downstream apoptotic pathway. No change in the levels of Bim was observed as it is not a target of E2F1. Elevated levels of apoptotic E2F1 targets were also seen in Hep3B cells ($p53^{-/-}$, with an integrated HBV genome), as compared with HepG2 cells (Fig. 4D), suggesting that E2F1 can induce its apoptotic targets in a p53-independent manner. To validate the apoptotic functions of E2F1 *in vivo*, we measured the levels of E2F1-specific apoptotic targets in the liver tissue lysates of 'X15-*myc*' transgenic mice. Unlike the upregulation observed for cell cycle and replication control proteins (Fig. 3A), no change in the levels of apoptotic targets of E2F1 was observed in the early (3-month old) liver samples. However, the tumour samples from 6-month-old mice showed a marked increase in the levels of apoptotic markers such as caspases and Apaf-1 (Fig. 4E). Thus, our results further define the role of E2F1 in unscheduled replication activity, DNA damage and apoptosis.

HBx modulates the intracellular levels of histone methyltransferase MLL1

To further elucidate the mutually exclusive functions of E2F1 in cell cycle progression and apoptosis, we investigated the involvement of selected co-activators in the upregulation of E2F1 target proteins. We observed the accumulation of MLL1 methyl transferase protein in the liver lysates of X15-*myc* transgenic mice of different ages (1, 3 and 6 months; Fig. 5A). Because MLL1 is also targeted by Skp2 for proteasomal degradation similar to E2F1 and Orc1, we speculated that Skp2–HBx interaction was involved in conferring intracellular stability to MLL1. Our immunoprecipitation studies suggested that MLL1 protein did interact with Skp2 in HepG2 cells but not in HepG2.2.15 cells, probably due to the sequestration of Skp2 by HBx (Fig. 5B). Together, these results suggested that deregulation of Skp2 functions by HBx can confer intracellular stability to MLL1.

Promoter occupancy of MLL1 seems to specify diametric functions of E2F1 in the HBx microenvironment

To understand the regulatory mechanism of the divergent cellular functions of E2F1, such as cell cycle progression and apoptosis, we analysed the stage-specific promoter occupancy of E2F1 on its target genes. Surprisingly, the oncogenic promoters MCM2 and MCM4 derived from transgenic mice of both age groups (3 and 6 months old) showed nearly similar E2F1 occupancy (Fig. 6A) independent of the target gene expression, which was exclusive to the young age group (Fig. 3A). Likewise, pro-apoptotic promoters (Casp-7 and Apaf-1) of both groups of transgenic mice showed nearly equal E2F1 occupancy (Fig. 6A) although their expression was seen only in 6-month-old mice (Fig. 4E). These results clearly indicated the consistent occupancy of E2F1 on its target promoters regardless of their gene expression. In contrast, the E2F1 co-activator protein MLL1 showed selective sequestration on E2F1 target promoters that engaged in their protein expression. This was evident from the marked increase in the MLL1 occupancy on MCM2 and MCM4 promoters derived from early hepatic tumours (of 3-month-old mice) that showed significant decline in late tumours (of 6-month-old mice). In sharp contrast, a low MLL1 occupancy was observed on caspase-7 and Apaf-1 promoters of early hepatic tumours that increased significantly in the late tumour microenvironment (Fig. 6B).

Conclusive to MLL1 occupancy, we observed the selective enrichment of histone H3 lysine 4 tri-methylation signature (H3K4Me3) on oncogenic (MCM2 and MCM4) and pro-apoptotic (Casp-7 and Apaf-1) promoters derived from early and late hepatic tumours, respectively (Fig. 6C). The transcription initiation marker phospho-Ser5 pol II also showed a similar pattern of occupancy on these promoters

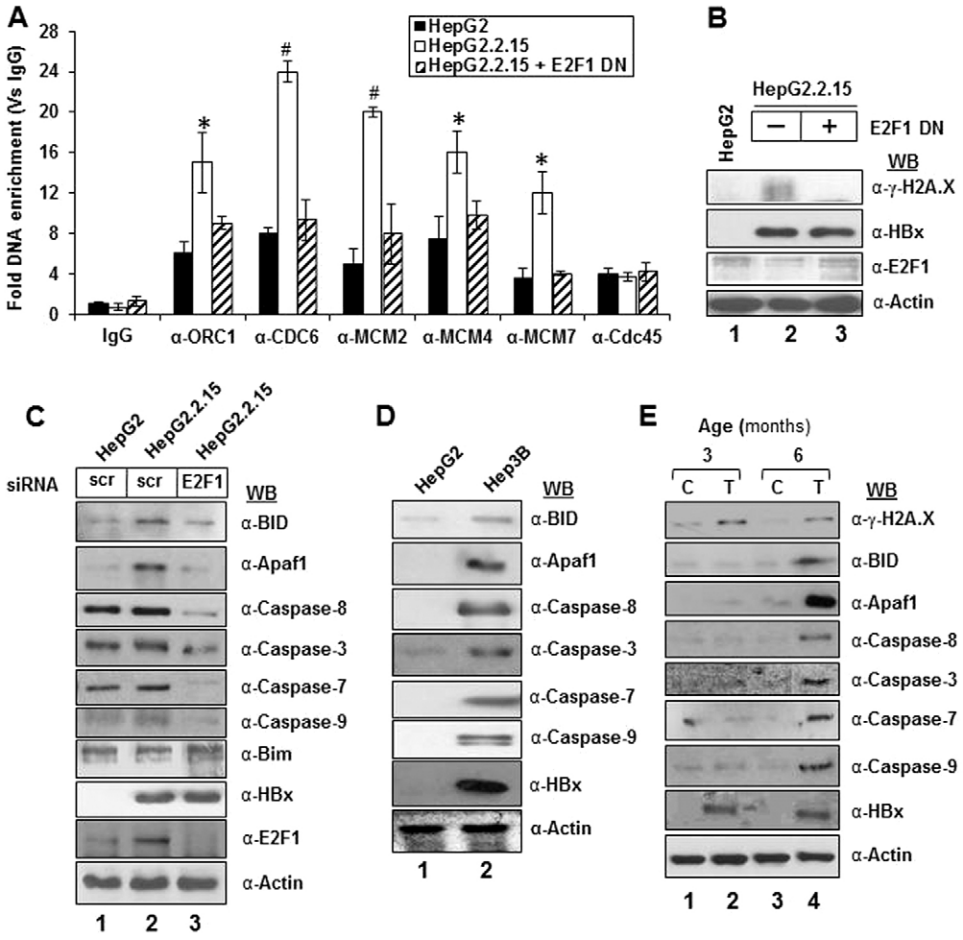

Fig. 4. Proliferative and pro-apoptotic activities of E2F1 in the presence of HBx. (A) ChIP-qPCR of lamin B2 origin in the lysates of HepG2 and HepG2.2.15 cells, untransfected or transfected with E2F1 DN construct to measure the occupancy of replication-associated proteins (ORC1, CDC6, MCM2, MCM4, MCM7 and Cdc45) over that of IgG control. Data are means ± s.d. of three independent experiments; $*P<0.05$ and $^{\#}P<0.01$. (B) Western blot analysis of the DNA damage biomarker γ-H2AX in the lysates of HepG2 or HepG2.2.15 cells either untransfected or transfected with E2F1 DN construct. (C) Western blot analysis of pro-apoptotic markers in the lysates extracted from scrambled (scr)-siRNA-transfected HepG2, HepG2.2.15 cells and E2F1-specific siRNA-transfected HepG2.2.15 cells. (D,E) Western blot analysis of the indicated pro-apoptotic markers in lysates of HepG2 and Hep3B cells (D) and liver tissue (E) of 3- and 6-month-old C57B6 control (C) and X15-*myc* transgenic (T) mice. Actin was used as loading control in these experiments.

(supplementary material Fig. S3). Corroborating results were also observed in HepG2.2.15 cells that showed a surge in MLL1 occupancy on MCM4 promoter after serum stimulation (Fig. 7A). However, the MLL1 occupancy shifted to the Apaf-1 promoter when cells were treated with cisplatin – chemotherapeutic drug known to induce apoptosis (Fig. 7B). As expected, no change in E2F1 occupancy was observed on MCM4 and Apaf-1 promoters (Fig. 7C,D). Remarkably, a more direct role of MLL1 in the regulation of E2F1 target promoters was evident from RNA interference studies. Silencing of MLL1 in HepG2.2.15 cells did not change the expression of E2F1, or its occupancy on target promoters. However, MLL1 silencing did affect the expression of E2F1 target proteins because of a lack of MLL1 occupancy on their promoters (supplementary material Fig. S4). Thus, the characteristic promoter occupancy of MLL1 could be a regulatory mechanism in specifying the dual complementary functions of E2F1.

The combinatorial activator/repressor complexes of E2F1 appear to regulate the promoter-specific sequestration of MLL1

Because the promoter occupancy of MLL1 seemed to specify the expression of E2F1 target proteins, we next investigated whether other E2F1 co-activator/repressor complexes had a role in the sequestration of MLL1 on the target promoters. Our immunoprecipitation studies suggested that the formation of the CBP co-activator complex increased substantially in the transgenic mice of both age groups. A similar increase in the Brg1 co-repressor complex was also observed. In contrast, no interaction was observed between E2F1 and co-activator GCN5 in the transgenic mice (Fig. 8A). Since E2F1 showed a promoter occupancy (Fig. 7C,D) and CBP reportedly interacts with MLL (Ernst et al., 2001), we examined the role of CBP in MLL1 occupancy on promoters with the help of RNA interference. When scrambled siRNA was used, CBP occupied MCM4 promoter during serum stimulation but was replaced by Brg1 following treatment with cisplatin (Fig. 8B). By contrast, the Apaf-1 promoter, which was initially occupied by Brg1, switched to CBP in the presence of cisplatin (Fig. 8D). However, the silencing of CBP expression led to the occupancy of only Brg1 on these promoters under both conditions (Fig. 8B,D). Further, knockdown of CBP prevented the occupancy of MLL1 on both MCM4 and Apaf-1 promoters without affecting the E2F1 recruitment (Fig. 8C,E). Apparently, the promoter occupancy of CBP and Brg1 correlated respectively with the activated and repressed states of these promoters. Note that the MLL1 protein level did not change after CBP knockdown (supplementary material Fig. S5A). However, unlike CBP silencing that downregulated MLL1 occupancy, the MLL1 gene silencing did not affect CBP occupancy on MCM4 and Apaf-1 promoters (supplementary material Fig. S5B,C). Together, these results suggested that E2F1 forms two mutually exclusive complexes with either CBP or Brg1. Furthermore, the formation of dynamic activator/repressor complex could regulate the sequestration of MLL1 on selective target promoters in a CBP-dependent manner.

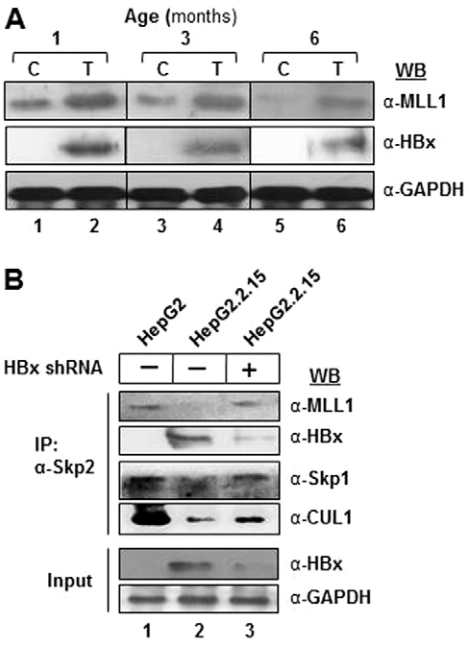

Fig. 5. Stabilization of MLL1 methyltransferase in the presence of HBx. (A) Liver tissue lysates from 1-, 3- and 6-month-old C57B6 control (C) and X15-*myc* transgenic (T) mice were western blotted for MLL1, HBx and GAPDH. (B) Lysates of HepG2 or HepG2.2.15 cells either untransfected or transfected with HBx shRNA were immunoprecipitated (IP) with anti-Skp2 and immunoblotted for MLL1, HBx and the SCF ubiquitin ligase complex proteins Skp1 and CUL1. One-tenth of the cell lysate was used to confirm the expression of HBx. GAPDH was used as loading control.

Discussion

The E2F family of proteins are well known to activate or repress genes involved in cell proliferation, differentiation and apoptosis (Dimova and Dyson, 2005). Their activity is regulated by multi-level networks that ensure tight control over context-specific functions. When deregulated, the increased activity of E2F1 cause inappropriate cell proliferation followed by apoptosis (DeGregori and Johnson, 2006), whereas a reduced proliferation capacity is related to a decreased activity (Wu et al., 2001). Mechanisms that regulate E2F activity include binding to Rb and p107, interaction with cylinA/cdk2, degradation by the ubiquitin–proteasome pathway and post-translational modifications including phosphorylation and acetylation. Of late, micro-RNAs as well as some co-activator and co-repressor proteins have also been reported to regulate E2F1 activity (reviewed by Wu et al., 2009). Although the above studies examined the molecular networks that regulate E2F activity, there is little information about E2F regulation by the ubiquitin–proteasome pathway.

The first evidence of E2F1 regulation by the ubiquitin–proteasome system came from the studies of its interaction with the E3 ubiquitin ligase Skp2, which targets E2F1 for ubiquitylation and degradation during the S–G2 phases of the cell cycle (Marti et al., 1999). Recent reports on E2F1 suggest that other E3 ligases also have a role in regulating E2F1 levels (Budhavarapu et al., 2012; Peart et al., 2010). Hijacking of ubiquitin ligases such as Skp2 by viral oncoproteins has been suggested as a new mechanism of tumourigenesis that may involve stabilization of some key regulatory proteins (Baresova et al., 2012; Kalra and Kumar, 2006). Here we report a new role of HBx oncoprotein in the post-translational regulation of E2F1 functions (Figs 1, 2). Interestingly, a similar role of HBx has also been reported for pituitary tumour transforming gene 1 which accumulates as a result of disruption of the SCF complex (Molina-Jiménez et al., 2010). Moreover, we found that the residual ubiquitylation of E2F1 in the HBx microenvironment may be conferred by APC/C complex because DP1 is known to specifically block the ubiquitylation and degradation of E2F1 by the APC/C complex (Fig. 2B). Interestingly, there is no evidence of a direct interaction between HBx and Cdc20 or Cdh1 – the substrate-recognition units of APC/C E3 ligases. Thus, HBx seems to perturb the intracellular pool of E2F1 by interfering with Skp2 functions, as other ubiquitylation pathway(s) were resistant to such deregulation.

E2F1 ubiquitylation and its binding to Rb have been considered to be mutually exclusive (Hofmann et al., 1996). Thus, the absence of E2F1 ubiquitylation in the presence of HBx was expected to modulate the E2F1–Rb axis by promoting Rb binding to E2F1. In contrast, we observed a massive increase in the hyperphosphorylated forms of Rb in the HBx microenvironment (supplementary material Fig. S1). Note that the phosphorylation of Rb is mediated by Cdk2 kinase, which is upregulated both at the transcriptional and post-translational levels in the presence of HBx (Mukherji et al., 2007; Singh et al., 2011). Thus, HBx could regulate E2F1 in two ways: (1) by promoting its intracellular abundance; and (2) by facilitating the accumulation of free active E2F1. In mammalian cells, the E2F family of transcription factors plays a pivotal role in the regulation of genes involved in the G1–S transition and DNA synthesis (Cam and Dynlacht, 2003). Moreover, E2F1 has the potential to function as an oncogene and regulates an important part of the circuitry that stimulates the cells to progress through the G1 phase, after which it is committed to complete the rest of the cell cycle (DeGregori et al., 1995). If appropriate signals are not received during cell cycle progression, a stress response ensues, driving the cell towards programmed cell death or apoptosis (Taylor et al., 2008). Indeed, the role of E2F in determining cell fate is not restricted to its effects on cell cycle progression as it can also efficiently induce apoptosis. In fact DNA damage is one of the earliest signals known to induce E2F1 apoptotic signalling in cells (Engelmann and Pützer, 2010). Although, the oncogene-induced apoptotic activity of E2F1 is well established under different conditions of deregulated E2F1 (Nahle et al., 2002), its role in stage-specific *in vivo* tumourigenesis is less appreciated. As we observed increased levels of E2F1 in the HBx microenvironment, we used the X15-*myc* transgenic mouse model to further investigate the molecular underpinnings of oncogene-induced apoptosis. This oncomouse model has been reported to develop HCC early in life, between 3 and 5 months of age, which lead to death at later stages (Lakhtakia et al., 2003). Analysis of E2F1 levels in the X15-*myc* transgenic mice showed an early accumulation of E2F1, beginning at 1 month of age (Fig. 1A). As HCC progressed (during the next 3 months), the expression of E2F1 target genes associated with DNA replication and cell cycle control, were elevated (Fig. 3A). The critical imbalance of the replication control proteins and rate-limiting factors such as Cdc45 seemed to trigger the replication-stress-induced DNA damage, which correlated with increased levels of γ-H2A.X (Fig. 4A,B). In response to DNA damage, the cells were sensitized to apoptotic

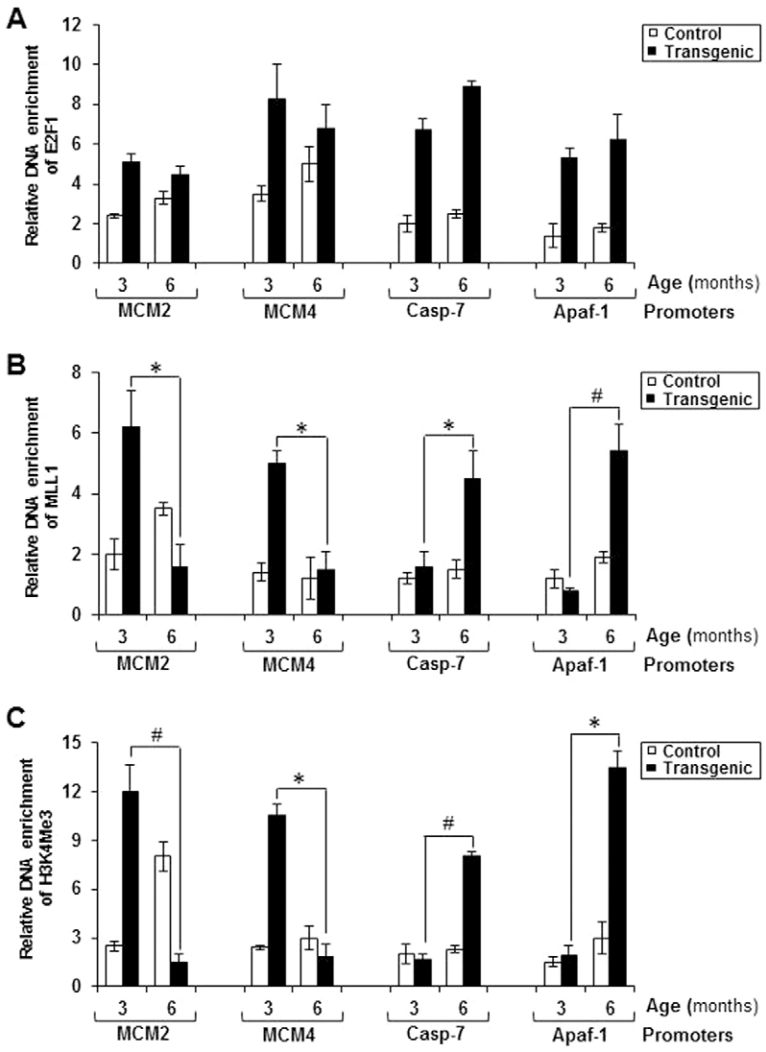

Fig. 6. Promoter occupancy of E2F1 and MLL1 proteins in transgenic mice of different ages. ChIP-qPCR analysis using liver tissue lysates from 3- and 6-month-old C57B6 control and X15-*myc* transgenic mice to show either the enrichment of E2F1 (A), MLL1 occupancy (B) or the enrichment of histone H3K4Me3 (C) over IgG control on oncogenic (MCM2 and MCM4) and apoptotic promoters (Casp-7 and Apaf-1). Data are means ± s.d. of three independent experiments; $^{*}P<0.05$ and $^{\#}P<0.01$.

death by the induction of apoptotic targets of E2F1 in the late tumour microenvironment (Fig. 4E). Intriguingly, neither the oncogenic nor the apoptotic target promoters of E2F1 showed stage-specific occupancy of E2F1 as evident from its continued enrichment on both promoters (Fig. 6A).

A number of co-regulators have been implicated in the downstream function of E2F1-mediated transcriptional regulation. These include HATs, HMTs and ATP-dependent chromatin remodelers (Blais and Dynlacht, 2007). The MLL-associated complexes have been reported to methylate histone H3 at K4 (Krivtsov and Armstrong, 2007) and the resulting H3K4me3 acts as a hallmark of transcription initiation at most genes (Guenther et al., 2007). In addition, the H3K4me3 marker may favour the promoter occupancy of MLL1 by its association with WDR5, a subunit of the MLL1 complex (Wysocka et al., 2005). Recent studies on E2F1 co-regulators involved in its downstream target activation suggested the recruitment of MLL1 to E2F-responsive genes either through direct mechanism or by host cell factor-1 (HCF-1) (Tyagi et al., 2007). Interestingly, just as E2F1, MLL1 protein is also a direct substrate for Skp2-mediated ubiquitylation and degradation (Liu et al., 2007) and, thus, got accumulated in the tissue lysates of transgenic mice (Fig. 5A). Moreover, in contrast to E2F1 occupancy, there was a shift in the promoter occupancy of MLL1 and the associated histone H3K4Me3 marker (Fig. 6). These changes seem to specify the expression of E2F1 targets as evident from the enrichment of transcription initiation marker (supplementary material Fig. S3). Thus, it is likely that before sensing the DNA damage, MLL1 occupied oncogenic promoters to modulate cellular proliferation (Fig. 7A). As soon as DNA damage is sensed, cells are sensitized to programmed cell death marked by a shift in the occupancy of MLL1 on apoptotic promoters (Fig. 7B).

Our investigations on the molecular events influencing whether E2F1 activates proliferation or apoptosis revealed a combinatorial mechanism of gene activation and repression. The E2F1 target promoters were characterized by the dynamic occupancy of co-activator CBP and co-repressor Brg1 (Fig. 8B,D). The interaction between E2F1 and CBP has been known for the past two decades (Trouche et al., 1996). In addition, a functional interaction between HBx and CBP has been recently reported that mediates CREB-related transcription (Cougot et al., 2007). HBx also might induce E2F1-dependent transcription through CBP interaction, as evident from the increased interaction between CBP and E2F1 the tissue lysates of X15-*myc* transgenic mice (Fig. 8A). However, unlike CBP, the

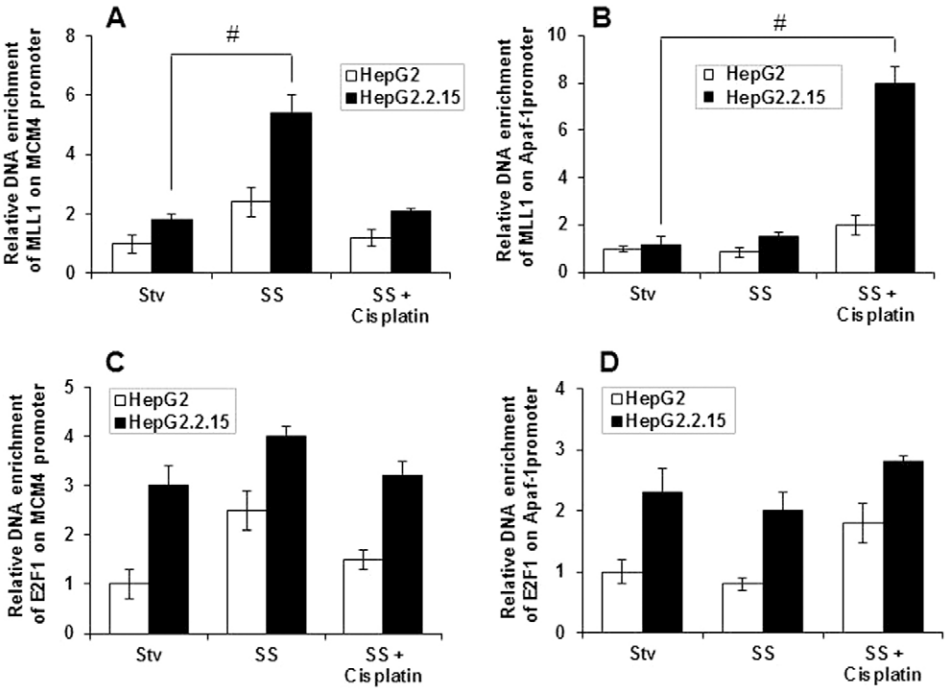

Fig. 7. Promoter occupancy of E2F1 and MLL1 proteins in hepatoma cells. HepG2 and HepG2.2.15 cells were either starved (Stv) or serum stimulated (SS) in the absence or presence of cisplatin and subjected to ChIP-qPCR analysis. (A,B) The enrichment of MLL1 occupancy, over IgG control, on the proliferative MCM4 promoter (A) or pro-apoptotic Apaf-1 gene promoter (B). $^{\#}P<0.01$. (C,D) Enrichment of E2F1 occupancy over IgG control on MCM4 (C) and Apaf-1 (D) gene promoters. Data are means ± s.d. of three independent experiments.

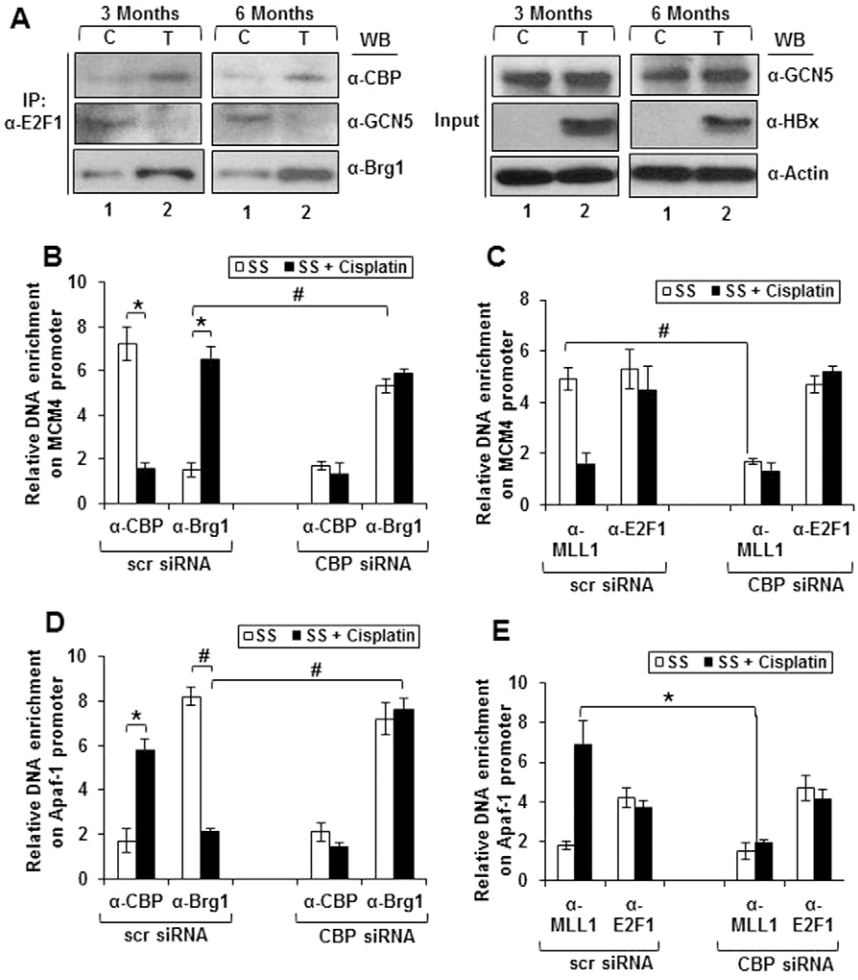

Fig. 8. Mutually exclusive promoter occupancy of E2F1 co-activator/repressor complexes in hepatoma cells. (A) Liver tissue lysates from 3- and 6-month-old C57B6 control (C) and X15-*myc* transgenic (T) mice were immunoprecipitated with anti-E2F1 and immunoblotted for CBP, GCN5 and Brg1. One-tenth of the tissue lysate was used to probe the expression of GCN5 and HBx. Actin was used as loading control. (B–E) HepG2.2.15 cells were transfected with either scrambled (scr) siRNA or CBP-specific siRNA. Cells were serum starved 24 hours post-silencing and subjected to serum stimulation (SS) either in the absence or presence of cisplatin (SS + cisplatin) and processed for ChIP-qPCR analysis. Enrichment of CBP and Brg1 occupancy over IgG control was measured on either MCM4 (B) or Apaf-1 gene promoters (D). Similarly, the same samples were used to measure the enrichment of MLL1 and E2F1 occupancy over IgG control on either MCM4 (C) or Apaf-1 gene promoters (E). Data are means ± s.d. of three independent experiments; $^{*}P<0.05$ and $^{\#}P<0.01$.

Brg1 interaction is facilitated by TopBP1 (Biswas and Johnson, 2012), which also mediates the GCN5–E2F1 interaction. The E2F1 complexes formed in the presence of TopBP1 represent the anti-apoptotic function of E2F1 since it facilitates DNA repair through ATR signalling and Chk1 activation (Guo et al., 2011; Liu et al., 2004). Note that HBx triggers the ATR damage signalling pathway in order to support viral replication but disables the downstream checkpoints signalling for the survival of host cells (Chaurushiya and Weitzman, 2009). These changes may account for the pro-apoptotic function of E2F1 as well as its basal interaction with GCN5 in the X15-*myc* transgenic mice (Fig. 4E; Fig. 8A). However, TopBP1-mediated recruitment of Brg1 to the E2F1 target promoters (Fig. 8B,D) may act as the transcriptional co-repressor of E2F1 activity, independent of its other cellular functions (Liu et al., 2004). Furthermore, our RNA interference studies suggested that the recruitment of MLL1 to the active target promoters was dependent on CBP (Fig. 8C,E) and was in agreement with the fact that MLL does interact with CBP (Ernst et al., 2001). Together, these data suggested the formation of a mutually exclusive E2F1 complex with either CBP or Brg1 to regulate the activator or repressor functions of E2F. In addition, the activator/repressor complex formation appeared to control the promoter-specific sequestration of MLL1 in a CBP-dependent manner. Cell signalling networks, such as PI3K/Akt signalling, may have a role in combinatorial complex formation in order to specify the dual functions of E2F1 (Hallstrom and Nevins, 2009). Interestingly, a recent report from our laboratory suggests that Akt-mediated phosphorylation of HBx can augment the oncogenic potential of this viral protein (Khattar et al., 2012). Further study would be necessary to understand the emergent molecular paradigms for the control of MLL1 activity in tumours.

Materials and Methods

Expression vectors and shRNA constructs

Construction of the eukaryotic expression vector for wild-type HBx (X0) has been described previously (Kumar et al., 1996). Expression constructs of DP1 (HA-DP1) and E2F1 DN [E2F1(1–374)] have been described elsewhere (Urist et al., 2004; Hsieh et al., 1997). Construction of HBx-specific small-hairpin RNA (HBx shRNA, X-D) has been reported earlier (Hung and Kumar, 2004).

Cell culture and transfection

Human hepatoma cell line Hep3B (ATCC HB-8064), hepatoblastoma cell line HepG2 (ATCC HB-8065) and its derivative HepG2.2.15 were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Transfection was carried out in a 60-mm culture dish (0.6×10^6 cells) with 2.0 μg of expression constructs using Lipofectamine (Invitrogen) according to the manufacturer's instructions. For RNA interference studies, 2.0 μg of HBx shRNA (X-D) was transfected using Fugene 6 (Invitrogen). siRNA against E2F1, MLL1 and CBP (all from Santa Cruz Biotechnology) were used for transfection according to manufacturer's instructions. Wherever indicated, 24 hours after transfection cells were serum-starved for another 24 hours and subjected to 8 hours serum stimulation either in the presence or absence of cisplatin.

Chemical inhibitors and antibodies

The chemical inhibitors were obtained from Sigma-Aldrich and were used at the following working concentrations: α-amanitin (5 μg/ml for 24 hours), cycloheximide (15 μg/ml either for 4 hours or for the indicated time periods), MG132 (50 μM for 4 hours) and cisplatin (25 μM for 8 hours). Antibodies were obtained from the following sources: Santa Cruz Biotechnology for E2F1, Ub, DP1, Skp2, Skp1, CUL1, Rb, P-Rb (Ser 795), cyclin E, Cdk2, ORC1, Cdc6, MCM2, MCM3, MCM4, MCM7, RPA, PCNA, Cdc45, Apaf1, caspase-7, caspase-8, MLL1, CBP, GCN5, Brg1, phospho-Ser5 pol II, actin, GAPDH and IgG; Cell signaling for γ-H2A.X [phospho-histone H2A.X (Ser139)], BID, Bim, caspase-3 and caspase-9; Upstate Biotechnology for histone H3 K4me3; and the monoclonal antibody (B-8/2/8) against HBx, which has been reported previously (Kumar et al., 1996).

Oncomouse model

Development of the transgenic mouse model (X15-*myc*) of HCC has been described earlier (Lakhtakia et al., 2003). All animal experiments were undertaken in accordance with the requirements of the Institutional Animal Ethics Committee. The animals were maintained under standard conditions, treated humanely and provided pelleted diet and water *ad libitum*. Control C57B6 and transgenic X15-*myc* mice were housed at 30–70% humidity and ~23℃). The tissue samples (liver) were surgically removed at the indicated age of the animals and processed for protein and chromatin immunoprecipitation studies.

Pulse-chase analysis

To determine the turnover of E2F1, both HepG2 and HepG2.2.15 cells were metabolically labelled in the presence of 110 μCi of [^{35}S]cysteine and [^{35}S]methionine mix (NEN Life Science Products) for 2 hours, followed by incubation with an excess of unlabelled methionine and cysteine for different time periods. E2F1 was immunoprecipitated from the cell lysates, resolved by SDS/PAGE (10% gel) and the bands were visualized using Phosphorimager.

Immunoprecipitation and western blot analysis

For immunoprecipitation, the lysates were incubated with the indicated primary antibodies at 4℃ and the immune complexes were isolated using protein-A–Sepharose (Amersham Biosciences). The samples were resolved using SDS-PAGE, electro-transferred to nitrocellulose membrane (Amersham Biosciences) and the protein bands were visualized by enhanced chemiluminescence (ECL) reagent (Cell Signaling Technology). For western blotting, equal amounts of protein samples were directly processed and visualized as above. The mouse liver samples were processed for western blotting as reported earlier (Lakhtakia et al., 2003).

Chromatin immunoprecipitation assay

ChIP assays were performed as per the manufacturer's instructions (Upstate Biotechnology). Briefly, 1×10^6 cells were cross-linked with formaldehyde (1%), lysed, and sonicated on ice (5 pulses at 30% amplitude) and centrifuged at 13,000 rpm for 10 minutes to obtain clear supernatant. Samples were pre-cleared for 2 hours with protein-A–Sepharose beads (Amersham Biosciences) and incubated overnight with indicated antibodies. The immune complexes were pulled down using protein-A–Sepharose beads. After a series of washing steps, DNA was eluted using QIAquick PCR purification kit (Qiagen), and analysed by SYBR green real-time quantitative PCR (qPCR). To study the divergent cellular functions of E2F1, both HepG2 and HepG2.2.15 cells were serum-starved for 48 hours. This was followed by 8 hours serum stimulation either in the presence or absence of cisplatin and the lysates were prepared as described above.

The liver samples for ChIP assays were processed as described elsewhere (Weinmann and Farnham, 2002). Briefly, 0.3 g of tissue in 10 ml of 1× PBS was cross-linked with formaldehyde (1%) and quenched with glycine. The tissue pieces were pelleted by centrifugation at 200 ***g***, washed with ice-cold 1× PBS and disaggregated with a polytron homogenizer. Cells were pelleted at 2000 ***g*** and resuspended in lysis buffer and the subsequent steps were the same as described for cultured cells.

SYBR green real-time quantitative PCR (qPCR)

The genomic DNA obtained by the ChIP assay was subjected to qPCR in triplicate with 1× PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6, containing SYBR Green at a final concentration of 0.5× and 5% DMSO) using the following primer sets: human lamin B2 origin, forward 5′-gactggaaacttttttgtac-3′, reverse 5′-gtggagggatctttcttagacatc-3′; mouse promoters of MCM2, forward 5′-gtggccataccgacacttaga-3′, reverse 5′-tttaccacgctaaggcaagg-3′; MCM4, forward 5′-ctgcagtagccaacaccgta-3′, reverse 5′-ctggttctgtccctttgcat-3′; Apaf1, forward 5′-cagcatcgcacacttaccag-3′, reverse 5′-tccagacatgtccgaagtga-3′; and caspase-7 forward 5′-agcgctctatctgcccagt-3′, reverse 5′-gaaagcaaaagtcccagagg-3′. In addition, primer pairs for human promoters of MCM4 and Apaf1 were also used. Data obtained were normalized with input DNA and expressed as either fold DNA enrichment or relative DNA enrichment over IgG control.

Statistical analysis

Data are expressed as means ± s.e.m. Means were compared by one-factor analysis of variance followed by Fisher protected least significant difference to assess specific group differences. Differences were considered significant at $P<0.05$.

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Author contributions

V.K. did study design, interpreted the results and refined the manuscript. M.S. and A.K.S. performed the experiments and drafted the manuscript.

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