The HBx protein of hepatitis B virus regulates the expression, intracellular distribution and functions of ribosomal protein S27a

Grace Fatima, Ganeshan Mathan† and Vijay Kumar

Virology Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi 110067, India

The pleiotropic HBx protein of hepatitis B virus is linked functionally to the development of hepatocellular carcinoma (HCC) via effectors and signalling pathways of the host. To identify such effectors in a macrocarcinogenic environment, a PCR-based cDNA subtraction analysis was carried out in the X15-myc oncomouse model of HCC. Altogether, 19 categories of genes, mainly involved in protein biosynthesis and the electron-transport chain, were found to be upregulated in the liver of these mice. Ribosomal protein S27a (RPS27a), which is a natural fusion protein of N-terminal ubiquitin and C-terminal extension protein (CEP), topped the list of expressed genes, with >20-fold higher expression compared with its normal level. Sustained and elevated expression of RPS27a in the mouse liver and its moderate expression in cell culture in the presence of HBx suggested an indirect role of RPS27a in hepatocarcinogenesis. Nevertheless, a remarkable change in the intracellular distribution of ubiquitin from cytoplasm to late-endosomal lysosomes, and of CEP from nucleoli to the perinucleolar region/nuclear foci, was observed in the presence of HBx. RPS27a accelerated the progression of the cell cycle and cooperated with HBx in this process. Further, the knockdown of RPS27a expression by RNA interference in an HBx microenvironment led to retarded cell-cycle progression and reduced cell size. Thus, these results suggest strongly that RPS27a could be an effector of HBx-mediated hepatocarcinogenesis.

INTRODUCTION

Chronic infection by hepatitis B virus (HBV) is a major public-health threat worldwide because of its reported association with cirrhosis, and more ominously hepatocellular carcinoma (HCC) (McMahon, 2009; Yang & Roberts, 2010). The oncogenic mechanisms of HBV have now begun to be elucidated. Multiple direct and indirect processes are involved in establishing HBV-related HCC (Kremsdorf et al., 2006). Integration of the HBV genome may stimulate tumorigenesis through either the cis-activation of cellular genes, such as the myc family of genes in woodchucks (Fourel et al., 1990), or trans-activation by the viral X protein or HBx (Caselmann, 1996). HBx has been studied extensively for its role in HCC. It is a pleiotropic trans-activator that can stimulate a wide range of viral and cellular promoters (reviewed by Benhenda et al., 2009; Kumar & Sarkar, 2004). The oncogenic potential of HBx is evident from its ability to modulate tumorigenic signalling pathways (reviewed by Benhenda et al., 2009; Bouchard & Schneider, 2004), upregulate the expression of cellular oncogenes such as c-myc and c-jun (Caselmann, 1996) and functionally inactivate the tumour-suppressor protein p53 (Ueda et al., 1995). Besides, HBx can transform immortalized cells in vitro (Höhne et al., 1990; Shirakata et al., 1989) and induce liver-specific tumours in transgenic mouse models (reviewed by Kumar, 2008). There is constitutive activation of selected signalling pathways that promote cell growth and survival (Chemin & Zoulim, 2009).

Although HBx stimulates a number of signalling pathways, the key host factors involved in tumorigenesis are rather poorly understood. Stimulation of the extracellular signal-regulated kinases (ERks) and the c-Jun N-terminal kinases (JNks) by HBx leads to the induction of members of the AP-1 family of proteins, which aid in cell-cycle progression (Benn et al., 1996; Khattar & Kumar, 2010; Nijhara et al., 2001). Further, HBx can directly modulate the activity of cdk2 and accelerate the cell cycle through a direct interaction with the cyclin E/A–cdk2 complex (Mukherji et al., 2007). HBx-mediated stimulation of quiescent cells has also been correlated with trans-activation of the c-myc proto-oncogene and inhibition of the p53, p21 and Sui1 proteins (Lian et al., 1999; Wang et al., 1994). HBx is also known to facilitate the binding and activation of several transcription factors that, in turn, stimulate the expression

1Present address: Department of Biomedical Science, Bharathidasan University, Truchirapalli, India.

A supplementary figure and table are available with the online version of this paper.
of RNA polymerase I–III-dependent genes (reviewed by Kumar & Sarkar, 2004).

Microarray analysis of HCC samples has shown up- and downregulation of many genes with redundancy in the pathways that promote cell proliferation (Okabe et al., 2001; Xu et al., 2001b). Subsequent studies suggested that some ribosomal proteins are overexpressed in HBV-associated human HCC tissues (Kim, 2006; Kim et al., 2004; Mathan et al., 2011). Enforced expression of ribosomal protein L36a is associated with increased cell proliferation and colony formation due to rapid cell cycling (Kim et al., 2004). Besides, ribosomal proteins are also reported to perform extra-ribosomal functions including DNA repair, transcription or even replication, which may aid the mitogenic function of cells (Warner & McIntosh, 2009; Yamamoto, 2000). Further, heterozygous mutations in many ribosomal protein genes of zebrafish can lead to elevated cancer susceptibility, suggesting their role as tumour suppressors (Amsterdam et al., 2004). In the present study, we have investigated the physiological significance of overexpression of the ribosomal protein S27a in an HBx microenvironment of a transgenic mouse model of HCC (Mathan et al., 2011).

RESULTS

Genes are differentially expressed in liver tumours of transgenic mice

To identify the natural effectors of HBx in host cells, total RNA from the liver tissue of control and X15-myc transgenic mice was extracted and subjected to a PCR-based cDNA subtraction analysis. High-throughput sequencing of the subtracted cDNA clones and analysis by BLASTN revealed that the upregulated mouse liver transcripts belonged to 19 categories, including 100 protein-biosynthesis genes, 81 electron-transport genes, 75 metabolism-related genes, 36 transport-molecule genes, 25 ubiquitin–proteasome pathway genes, 23 protein-metabolism genes, etc. (Table 1), suggesting that the subtraction library consisted of a complex population of expressed genes. The classes of differentially expressed genes involved in diverse biological processes can be seen in Supplementary Table S1 (available in JGV Online). The highly expressed genes belong to two major groups of proteins involved in either protein biosynthesis or the electron-transport chain. Of these, ribosomal protein S27a (RPS27a), belonging to the protein-biosynthesis group, exhibited the highest expression level (overexpressed 20-fold), followed by other genes including cytochrome c oxidase subunits II and III, ATP synthase and NADH dehydrogenase (Table 2). For this reason, the subsequent studies were focused on understanding the role of the RPS27a gene.

**The RPS27a gene is expressed early and throughout the life of transgenic mice**

RPS27a is a 156 aa long, highly basic natural fusion protein with an N-terminal ubiquitin moiety and a C-terminal

<table>
<thead>
<tr>
<th>Category</th>
<th>Frequency (no. of genes)</th>
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<tbody>
<tr>
<td>Protein biosynthesis</td>
<td>100</td>
</tr>
<tr>
<td>Electron transport</td>
<td>81</td>
</tr>
<tr>
<td>Metabolism-related</td>
<td>75</td>
</tr>
<tr>
<td>Transport molecules</td>
<td>36</td>
</tr>
<tr>
<td>Ubiquitin–proteasome pathway</td>
<td>25</td>
</tr>
<tr>
<td>Protein metabolism</td>
<td>23</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>22</td>
</tr>
<tr>
<td>RNA processing</td>
<td>18</td>
</tr>
<tr>
<td>Cytoskeletal organization</td>
<td>17</td>
</tr>
<tr>
<td>Calcium/sugar/carbohydrate/copper binding</td>
<td>14</td>
</tr>
<tr>
<td>Endocytosis/protein transport</td>
<td>13</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>13</td>
</tr>
<tr>
<td>Transcription</td>
<td>13</td>
</tr>
<tr>
<td>Cell cycle and growth differentiation</td>
<td>12</td>
</tr>
<tr>
<td>Immune response</td>
<td>11</td>
</tr>
<tr>
<td>Apoptosis and anti-apoptosis</td>
<td>9</td>
</tr>
<tr>
<td>Blood coagulation</td>
<td>9</td>
</tr>
<tr>
<td>Molecular chaperones</td>
<td>8</td>
</tr>
<tr>
<td>Replication</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 1. Proteins overexpressed in the livers of X15-myc transgenic mice**

human ubiquitin carboxyl extension protein 80 (HUBCEP80) of 80 aa. It is a constituent of the small (40S) ribosomal subunit and has a role in the regulation of cell growth (Monia et al., 1989; Redman & Rechsteiner, 1988, 1989). As RPS27a is one of the highly expressed genes in the liver of X15-myc mice, its temporal expression was examined in animals of different age groups. Immunohistochemical (IHC) analysis of paraffin-embedded liver sections showed a diffuse cytoplasmic and nuclear expression in the early stage (1 month) of tumour development (Fig. 1a). At 6 months of age, a heavy nuclear–perinucleolar expression of RPS27a was observed (Fig. 1b) whereas, during the late stages of HCC development (18 months), a diffuse pattern of RPS27a staining was observed both in the cytoplasm and nucleus. The IHC index of RPS27a (Fig. 1c) showed that approximately 95 % of hepatocytes were positive for RPS27a in the first month of the life of control mice; this later declined dramatically to almost-undetectable levels and stayed like that throughout the life of the mice thereafter. In contrast, in the X15-myc mouse liver, approximately 50 % of the hepatocytes stained positive for RPS27a in the first month of the life of control mice; this later declined dramatically to almost-undetectable levels and stayed like that throughout the life of the mice thereafter. In contrast, in the X15-myc mouse liver, approximately 50 % of the hepatocytes stained positive for RPS27a in the first month, increasing to approximately 80 % within 6 months and then declining to approximately 30 % during the late stages (18 months) (Fig. 1c). These results, nevertheless, suggest that RPS27a is present throughout tumour progression in the transgenic mice.

**The RPS27a gene is a transcriptional target of HBx**

HBx is a well-known promiscuous trans-activator of many functionally important genes. To find out whether the RPS27a gene is a transcriptional target of HBx's, levels of the RPS27a transcript were measured by quantitative (q)PCR
analysis in an HBx microenvironment. A statistically significant 2.3-fold increase in RPS27a mRNA levels was observed in the presence of HBx (2.31 ± 0.41; P<0.001).

Further, a significant 3.1-fold increase in reporter-gene activity was also observed when the RPS27a-CAT reporter plasmid was co-transfected with the HBx expression vector (3.12 ± 1.36; P<0.001). Thus, the RPS27a gene appears to be a transcriptional target of viral HBx.

**Relocalization of the ubiquitinS27a and CEP moieties of RPS27a in the presence of HBx**

As HBx was found to stimulate expression of the RPS27a gene, we next examined the intracellular localization of the N- and C-terminal moieties of RPS27a in the presence of HBx. Confocal microscopy studies showed that the N-terminal ubiquitinS27a region is scattered in the cytoplasm, whilst the C-terminal CEP region is accumulated in the nucleolus (Fig. 2a). Co-expression of HBx and RPS27a (Fig. 2b) resulted in relocalization of the CEP moiety to the perinucleolar areas (arrow) and specific foci in the nucleoplasm (arrowhead). Interestingly, ubiquitinS27a and HBx were found to be co-localized in the cytoplasm (Fig. 2c).

To establish the association of HBx–ubiquitinS27a complexes with a specific cell organelle, cells co-expressing HBx and RPS27a were harvested in the presence of lysosomal tracker and analysed by confocal microscopy. In of Fig. 2d(i–iv), HBx is shown in green, ubiquitinS27a is pseudo-coloured in blue and the late-endosomal tracker is shown in red. The corresponding merged images in different combinations are shown in Fig. 2d(v–vii). We observed that HBx was co-localized with ubiquitinS27a; all ubiquitinS27a was associated with HBx, but not all HBx was associated with ubiquitinS27a [Fig. 2d(v)]. Further, HBx was seen primarily in the late endosomes, as evident from lysosomal tracker [Fig. 2d(vi)], whereas ubiquitinS27a was found both inside and outside late endosomes [Fig. 2d(vii)]. Thus, the HBx–ubiquitinS27a complexes are localized in late-endosomal lysosomes.

**Co-expression of RPS27a and HBx leads to improved survival and proliferation of cells**

As RPS27a and HBx are well-known to have an important role in cell growth, their cooperation in cell survival was examined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay in the presence of an RPS27a antisense construct (RPS27a−). The results were plotted against those of control cells (taken as 100 %). We observed that cells transfected individually with HBx or RPS27a exhibited a moderate (10 %) increase in cell survival compared with

**Table 2. Five highly expressed genes in the livers of X15-myc transgenic mice**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Level of overexpression (fold)</th>
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<tbody>
<tr>
<td>Ribosomal protein S27a</td>
<td>RPS27a</td>
<td>20</td>
</tr>
<tr>
<td>Cytochrome c oxidase (subunit III)</td>
<td>Cox3</td>
<td>16</td>
</tr>
<tr>
<td>ATP synthase (subunit 6)</td>
<td>ATP6</td>
<td>14</td>
</tr>
<tr>
<td>Cytochrome c oxidase (subunit II)</td>
<td>Cox2</td>
<td>11</td>
</tr>
<tr>
<td>NADH dehydrogenase (subunit 1)</td>
<td>ND1</td>
<td>11</td>
</tr>
</tbody>
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**Fig. 1.** Temporal expression of the RPS27a gene in X15-myc transgenic mice. (a) IHC detection of RPS27a in the liver of 1-, 6- and 18-month-old control and transgenic mice (at x200). (b) Liver nuclei of 6-month-old transgenic mice (at x800) showing heavy accumulation of RPS27a in the nuclear and perinucleolar areas. (c) Bar graph showing percentage of hepatocytes immune-stained for RPS27a expression in control mouse (black bars) and transgenic mouse (white bars). Cells (n=500) were counted randomly from 10 different areas. *P<0.01; **P<0.05.
control cells, whereas a significant (40 %; \( P < 0.001 \)) increase in cell viability was observed in the presence of both HBx and RPS27a (Fig. 3a). Selective knockdown of RPS27a by RNA interference did not affect cell growth. However, knockdown of RPS27a in an HBx microenvironment caused a 30 % decrease in cell viability. Thus, HBx and RPS27a seem to cooperate in maintaining cell growth/survival.

Next, we examined the effect of HBx/RPS27a-mediated cell growth on the cell cycle. Flow-cytometry analysis suggested that there was no major difference in the G0/G1, S and G2/M populations of cells transfected with HBx or RPS27a compared with respective controls. However, co-expression of HBx and RPS27a resulted in more cells (18.1 %) in the S phase and fewer cells (16.2 %) in the G2/M phase compared with controls. Interestingly, knockdown of RPS27a by RPS27a in an HBx microenvironment led to the accumulation (71 %) of cells in the G0/G1 phase (Fig. 3b). Thus, RPS27a appeared to provide a proliferative advantage to cells expressing viral HBx.

**RPS27a regulates the cell-size checkpoint in the presence of HBx**

Earlier observations of RPS27a-mutant cells in *Saccharomyces cerevisiae* have hinted at the role of RPS27a in ribosomal...
biogenesis and cell growth (Finley et al., 1989; Monia et al., 1990). Further, a dynamic transcriptional network involving activators of ribosomal proteins is known to increase ribosome synthesis and cell size (Finley et al., 1989; Jørgensen et al., 2004). For these reasons, the reduction in proliferation and cycling potential of HBx in an RPS27a-knockdown environment and its relation to cell size were analysed further. The mean forward scatter height (FSC-H) of cells during the cell cycle was plotted to show the relative size of cells during each phase of the cell cycle. The reduction in cell size was less prominent for cells expressing either HBx, RPS27a or both. Further, the reduction in cell size was only minimal in RPS27a-knockdown cells, suggesting that RPS27a has a role in cell growth that can either be supplemented by reducing the amount of protein or be rescued by some other regulatory molecules. However, there was a dramatic reduction in cell size when RPS27a gene expression was knocked down in an HBx microenvironment (Fig. 3c).

Besides, such a conditional reduction in cell size was observed during all three phases of the cell cycle (Fig. 3d). Thus, these results reiterated the need for RPS27a in an HBx microenvironment primarily for maintaining cell size, and thus regulating the proliferation of cells.

**DISCUSSION**

Increasing evidence suggests that HBx of HBV is a multifunctional protein that targets various pathways of the host system, as also supported by strategies of different viral proteins. The transcriptomic profiling of human HCC tumours has suggested that the differentially expressed genes may have a major role in HCC (Chen et al., 2002; Gravel et al., 2001; Okabe et al., 2001; Shirotta et al., 2001; Xu et al., 2001a, b). These reports suggest that there are multiple pathways to develop HCC with inbuilt redundancy.
transgenic environment created by HBx expression in the mouse liver seems to mimic a perfect macroenvironment for the development of HCC with a human carcinogenic allegory. Interestingly, some of the genes that are overexpressed in mouse liver cancer have been found to be overexpressed in some human tumours. For example, the RPS27a or HUBCEP80 gene has been reported to be overexpressed in fibroadenomas of breast (Adams et al., 1992), colorectal (Wong et al., 1993) and renal (Kanayama et al., 1991) cancers. It seems that, besides imparting a role in ribosome biogenesis and post-translational modifications of proteins, RPS27a could perform extra-ribosomal functions in concert with other overexpressed genes (Table 1), including transcription, RNA splicing, translation, cell cycle, metabolism, protein folding and degradation, apoptosis, immune response, metal binding, etc. (Warner & McIntosh, 2009; Wool, 1996).

In the present study, RPS27a was identified as an overexpressed gene in the tumour tissue (liver) of X15-myc transgenic mice (Table 2). We observed a high tissue expression of RPS27a from early to terminal stages of tumour development and progression. Importantly, the IHC index of RPS27a revealed that it had high expression in normal liver tissue, but declined sharply later on (Fig. 1a, c). This may be because hepatocytes enter the quiescent stage by the second or third month and thus may have a minimal requirement for ribosomal proteins. However, in X15-myc mice, the hepatic expression of RPS27a remained steadily higher until the terminal stages (18 months of age) of animals (Fig. 1c). This can be extrapolated from the fact that rapidly dividing hepatocytes in HCC tissue may have a higher metabolic rate than normal liver cells, and persistently high levels of RPS27a could contribute towards enhanced ribosome synthesis and protein turnover. Moreover, treatment of X15-myc mice with a plant extract bearing anti-cancer properties abolished the expression of RPS27a; these studies not only emphasized the involvement of RPS27a in hepatocarcinogenesis, but also indicated a putative prognostic value of this protein (Mathan et al., 2011).

Our transient transfection studies suggested that HBx can stimulate the expression of endogenous RPS27a mRNA to a statistically significant level. This was further substantiated by reporter-gene studies that showed the induction of the RPS27a promoter in the presence of HBx. Thus, besides normal physiological regulation, the RPS27a gene can be modulated by a regulatory viral protein and thus may have a role in carcinogenesis. Besides imparting a regulatory role, HBx also affected the intracellular distribution of RPS27a in Huh7 cells. The CEP region of RPS27a was targeted to perinucleolar compartments (PNCs) and foci in the nucleoplasm (Fig. 2b) in the presence of HBx, suggesting that RPS27a could be an effector molecule. Note that PNCs are mostly found in transformed cells whose function is largely unknown. However, the presence of heteronuclear ribonuclear proteins, splicing factors and small RNAs (Ghetti et al., 1992) in PNCs suggests a role of HBx in RNA processing (Wang et al., 2003). As CEP is reported to form an RNA–protein complex (Redman & Reichsteiner, 1989), its involvement in the formation of ribosomes and processing of rRNAs, especially in an HBx microenvironment, needs further investigation. Interestingly, the livers of adult X15-myc mice also exhibited a predominantly perinucleolar/nuclear distribution of the RPS27a protein (Fig. 1b), perhaps reflecting the active state of ribosome biogenesis.

Viral proteins are well-known to manipulate the functions of the ubiquitin–proteasome system to facilitate virus replication and to evade immune surveillance (Masucci, 2004). They not only manipulate the enzymes of the system to accomplish the desired actions, but also bind to the ubiquitin–proteasome machinery for this purpose. HBx is known to bind to the 20S α-subunit and 19S regulatory component and to inhibit the proteasomal machinery from performing its physiological functions, thereby inhibiting its own degradation (Hu et al., 1999). Moreover, the proteasome function is required for the trans-activation function of HBx. We observed that HBx colocalized with ubiquitinS27a; the colocalization increased with time and was targeted to late-endosomal lysosomes (Fig. 2c). As the lysosomal targeting of HBx has not been reported in any previous localization studies (Henkler et al., 2001), it could be an important regulatory mechanism to control HBx levels in cells. Late endosomes are primarily involved in the proteolysis of intracellular membrane proteins from the Golgi and extracellular proteins as a defence mechanism (Piper & Luzio, 2007). Although ubiquitin–dependent proteasomal degradation of HBx has been proposed (Hu et al., 1999), its proteolysis through the endosomal pathway is a distinct possibility.

The observation that RPS27a promoted cell growth just like HBx and supported improved growth when co-expressed with HBx suggested a cooperation between these two regulatory proteins. Apparently, RPS27a-mediated cell growth was related to the increase in cell-cycle progression. RPS27a is necessary to execute the oncogenic potential of the cell, as evident from the reduction in the proliferative capacity of cells by RNA interference (Fig. 3a). A report on the up- and downregulation of different subsets of ribosomal proteins in the presence of HBx is already available (Kim et al., 2001). Still, no physiological or pathological relevance or consequence is known. One of the fascinating features of oncogenicity is not only unregulated proliferation of cells, but also maintenance of the cell size or volume. Regulation of cell size has been largely ignored for several decades now, as opposed to the extensive literature on cell proliferation (Echave et al., 2007). Emphasis and work on cell growth came into the limelight only a few years back, with the discoveries of several key players playing important roles in maintaining the cell and thus the organ size. UB13 deletion mutants in S. cerevisiae were abnormally sized (Finley et al., 1989). A knockdown of RPS27a in an HBx microenvironment, although it reduced the size of the cells only marginally,
clarified the role of RPS27a in maintaining cell size. HBx also reduced the size of the cells, but again only marginally. However, reducing the expression of RPS27a in the presence of HBx caused a dramatic reduction in cell proliferation and size (Fig. 3c). To remain constant in size under proliferative conditions, cells must double in mass through increased macromolecular synthesis and grow to increased size during each cell-division cycle. Moreover, sufficient cell growth is required for cell-cycle progression but not vice versa, leading to the postulation of a cell-growth checkpoint for cell-cycle progression (Johnston et al., 1977). Such an HBx microenvironment with reduced protein load resulted in a blockade at the G0/G1 phase (Fig. 3b). Our finding that RPS27a-knockdown cells in an HBx microenvironment proliferate less at a smaller size suggests that RPS27a is necessary for maintaining the size of the cell. This necessity is underplayed in a normal scenario because, in the absence of HBx, neither the size nor the proliferation of the cells is compromised (Fig. 3c, d). A sustained proliferative response therefore requires coordination of both cell-cycle progression and cell growth (increase in cell size and mass) (Neufeld & Edgar, 1998). This coordination is breached in the absence of RPS27a and is inflated in the presence of HBx, with accelerated proliferation of cells. Moreover, to substantiate it further, the proliferative potential of HBx is enhanced by the constitutive expression of RPS27a. The molecular mechanisms of the cell-size pathway are currently under study.

HBx as an accelerator of cell proliferation also needs a machinery that will not compromise the growth of the cells. Alteration of RPS27a functions by HBx might be associated with creation of a permissive environment for HCC, which answers the need for an upregulation of RPS27a in HBx-related HCC. Thus, this appears to be yet another example of a virus (HBV in this case) that has devised mechanisms to use the cells’ own housekeeping machinery to circumvent the checkpoint and to make them proliferate unchecked. Hence, exploration of and targeting this pathway in great detail might lead to therapeutic intervention for HBV-related HCC.

**METHODS**

**Expression vectors and reporter genes.** The HBx expression vector is described elsewhere (Kumar et al., 1996). The β-galactosidase expression plasmid pCH110 (GE Healthcare) was used as transfection control. An expression vector of the human RPS27a gene for confocal experiments was developed by cloning a PCR-amplified fragment. The primers used were: forward (RPS27a-HA-F), 5'-GGATCCATGTACCCCATACGTTGCTCAGTATTAGCTCAGAT-TTTGTGAAAC-3'; reverse (RPS27a-HA-R), 5'-AGATCTCTTGCTCTGGTTTGTTG-3', to acquire a haemagglutinin (HA) tag at the N terminus of the RPS27a protein; reverse (RPS27aASR), 5'-AGATCTCTTGCTCTGGTTTGTTG-3', being complementary to the C terminus of the HUBCEP80 gene, but without a stop codon to acquire a hexahistidine tag from the vector backbone. The 494 bp PCR amplicon was initially cloned into the pGEM-T Easy vector and then subcloned into the BamHI site of the pCDNA3.1/myc-His(–)A mammalian expression vector (both from Invitrogen). The HA and hexa-His tags served to detect the ubiquitinating RPS27a, and CEP regions after cleavage. The expression of HA–RPS27a–pcDNA was confirmed by Western blotting after transient transfection in mammalian cells (see Supplementary Fig. S1, available in JGV Online). The native RPS27a recombinant (RPS27a) and its antisense complement (RPS27a−) were generated by PCR using the following set of primers: forward (Ubi-F), 5’-CGAGATCTATGCAGATTTTCGTGAAAACC-3'; reverse (CEP-R), 5’-TCGGATCCATCTGTTCTCTGGT-3'; and the above RPS27a cDNA template. The pRPS27a-CAT (chloramphenicol acetyltransferase) reporter construct was generated by cloning a 1.6 kb promoter region of the human RPS27a gene between the BglII/BamHI sites of the pCAT3basic vector (Promega). The promoter region was PCR-amplified using human genomic DNA and the following set of primers: P1F, 5’-CTCTCGAGATCTAGGATC-3'; P2R, 5’-TCTATCGGATCCAGGTTTCAACC-3’.

**Chemicals, enzymes and antibodies.** Propidium iodide, DMSO, MTT and methanol were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM), FBS, streptomycin and penicillin were from Life Technologies. All restriction enzymes were from New England Biolabs. Specificity of the mAb (B-8/28) against HBx has been reported previously (Kumar et al., 1996). An RPS27a mAb (M01) was procured from Abnova, while anti-mouse and anti-rabbit antibodies conjugated to Alexa Fluor 594 and 488 were from Invitrogen. All other antibodies were purchased from Santa Cruz Biotechnology, Inc.

**Cell culture and DNA transfection.** The human hepatoma HuH7 cell line (Nakabayashi et al., 1982) was maintained in DMEM supplemented with 10% FBS, penicillin (100 μg ml−1) and streptomycin (100 μg ml−1). Cells were transfected with different plasmids in a 60 mm culture dish (5 × 105 cells) using Lipofectamine as per the manufacturer’s instructions (Invitrogen) and studied for different parameters. The transfection efficiency was between 60 and 70%, as monitored with the help of EGF expression by cotransfecting 0.5 μg pEGFP-C1 plasmid (Clontech).

**Cell-viability assay.** The MTT assay was performed as described previously (Choeden et al., 2010).

**CAT assay.** The CAT assay was performed as described by Bocquel et al. (1989). Briefly, β-galactosidase activity was determined using an equal amount of protein from each cell extract, while CAT activity was measured using normalized amounts of protein in cell extracts. The autoradiographs were analysed by densitometry and the data were evaluated statistically by Student’s t-test.

**RNA isolation and real-time qPCR.** Total RNA was isolated from cells using TRIzol reagent as per the supplier’s instructions (Life Technologies). The levels of RPS27a mRNA were determined by qPCR (Schmittgen & Livak, 2008) using the following primers: forward (RPS27a1F), 5’-AGGGTGACCTGCTGGTCTCG-3'; reverse (RPS27a1R), 5’-AGATCTCTTTCCTCCTCTCGTTGTTG-3' in 1 × PCR buffer (50 mM KCl, 1.5 mM MgCl2, 10 mM Tris/HCl, pH 7.6, containing 0.5 × SYBR Green and 5% DMSO). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control using the following primers: forward, 5’-CGGCCATTATTGACCACTGCTC-3'; reverse, 5’-AGGGTCTACATGGCAAACGTTG-3'. The amplicons sizes for RPS27a and GAPDH were 480 and 228 bp, respectively. The transcript levels were analysed by the comparative Ct method (Schmittgen & Livak, 2008).

**Flow cytometry and immunofluorescence.** Flow cytometry and cell-cycle analysis were done as described previously (Mukherji et al., 2007). Briefly, cells were washed with PBS, fixed in 70% ethanol and stained with propidium iodide (50 μg ml−1). The cell suspension was analysed with a FACScan flow cytometer (BD Biosciences). The percentage of cell-cycle distribution was determined by using FlowJo software.
(TreeStar). The mean FSC-H was determined as a measure of relative cell size.

For immunofluorescence staining, cells were seeded on coverslips in 12-well culture plates, transfected as described above and harvested after 36 h. After a PBS wash, cells were fixed with 2% paraformaldehyde for 15 min and permeabilized with 0.5% Tween 20 for 10 min at room temperature. After rehydration in PBS for 30 min, cells were incubated for 1 h at room temperature with appropriate antibodies in PBS containing 5% BSA. After a quick wash, cells were incubated further for 1 h either with anti-mouse IgG coupled to Alexa Fluor 488 dye and/or with anti-rabbit IgG coupled to Alexa Fluor 594 dye in PBS with 5% BSA at a 1:200 dilution. After a PBS wash, the coverslips were mounted in anti-fade reagent containing DAPI (Invitrogen); the fluorescence images were acquired at ×60 using a Nikon AIR confocal microscope and analysed with the help of NIS-Elements software (Nikon).

Animal tumour model liver IHC. Development and maintenance of the transgenic mouse model of HCC (X15-myc) has been described previously (Choe et al., 2010). Expression of RPS27a was investigated in the liver of these mice by IHC using the avidin–biotin complex (ABC) method (Brathauer, 1994). Briefly, 2 μm thick paraffin sections of liver on glass slides were dewaxed in xylene, rehydrated, treated with 3% hydrogen peroxide and processed for antigen retrieval in 10 mM citric buffer (pH 6.0) in a boiling water bath for 15 min. After cooling, the sections were blocked with goat serum for 20 min, incubated with mouse anti-RPS27a (at a 1:300 dilution in PBS and 1% BSA) and processed for IHC analysis using the Dako Cytomation-LSAB system and an HRP kit (Dako) as per the manufacturer’s protocol. The sections were then counterstained with haematoxylin and dehydrated and mounted with DPX (BDH Laboratory Supplies). In control samples, the incubation step with the primary antibodies was omitted. The labelling index was determined by counting the number of brown-stained cells by two independent observers. At least 500 cells from different areas of a slide were visualized by light microscopy. The mean percentage of positive cells was plotted against respective control samples.

Subtraction cDNA library. The subtraction cDNA library was constructed using a unique mRNA pool expressed in the hepatic tumours of 6-month-old X15-myc transgenic mice. Liver tissue from X15-myc and C57BL6 mice of matched age was homogenized in TRIZol (1:10 ratio) and spun at 11000 g to obtain supernatant. After a brief extraction with chloroform, the supernatant was precipitated with 2-propanol to obtain total RNA. The poly(A) mRNA population was isolated by magnetic separation after annealing with biotinylated oligo-dT primer and immobilizing it onto streptavidin-linked paramagnetic beads. The bound mRNA was recovered in DEPC-treated water and used for library construction. First-strand cDNA synthesis was done using control mRNA (driver) isolated from the liver of C57/B6 mice with the help of oligo-dT primer and SuperScript II RNase H−reverse transcriptase. The driver was hybridized with the tester mRNA from the X15-myc mice (in a 5:1 ratio) at 65°C for 4–6 h in 10 mM Tris/HCl buffer containing 500 mM KCl. The tester–driver hybrids and excess of the driver were separated from the unhybridized tester poly(A) mRNA by magnetic separation. The tester mRNA that did not hybridize with the driver was used for constructing the cDNA library using a ZAP-cDNA Gigapack III Gold cloning kit (Stratagene).

Isolation of phage clones and PCR screening. Plaques were grown on NZY agar plates at 37°C for 6–8 h as per the supplier’s protocol (Stratagene). Individual plaques were picked randomly and transferred to microfuge tubes containing 500 μl SM buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM MgSO4) with 0.3% chloroform and 7% DMSO and stored at −80°C until used. Positive clones were identified by PCR using 5 μl phage extract in SM buffer and M13 phage-specific forward and reverse primers: M13F, 5’-GTTGTTAAAGCAGCGCAGTG-3’; M13R, 5’-CACAGGAA- ACAGCTATGACCATG-3’, in a 100 μl final mix in 96-well plates. Sequence information on 500 clones with >350 bp insert size was acquired using a DYEnamic ET Dye Terminator Cycle Sequencing kit and the high-throughput MegaBACE DNA Analysis system (Amersham Biosciences).

Analysis of DNA sequence data. Vector sequence from the sequencing data was removed by using the available vector-screening program from NCBI (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Sequence homologies were analysed in GenBank/EMBL using the BLASTN program (http://blast.ncbi.nlm.nih.gov/BLAST.cgi) (Altschul et al., 1997). Frequently appearing sequences were aligned with the original sequence by using the Nucleotide program (http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi).

Statistical analysis. Results are expressed as means ± SD and all statistical comparisons were made by one-way Student’s t-test; P-values ≤ 0.05 were considered significant.

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