Supplementary Information for:

Comparative analysis of SV40 17kT and LT function *in vivo* demonstrates that LT's C-terminus re-programs hepatic gene expression and is necessary for tumorigenesis in the liver

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Supplementary Figure Legends

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Figure S2. PCR-based strategy for mapping and sequencing of spliced early viral transcripts in livers of ApoE-rtTA$_{M2}$:TRE2-TAg transgenic mice. (Adobe pdf file .pdf)

Figure S3. RT-PCR analysis and sequencing of viral transcripts expressed in livers of ApoE-rtTA$_{M2}$:TRE2-TAg transgenic mice showing that line 6-1 expresses 17kT and st, but not LT. (Adobe pdf file .pdf)

Figure S4. Histopathology (H&E) and analysis of proliferation (PCNA) in livers of mice expressing 17kT or LT. 17kT and LT both promote hyperplastic liver growth, but only LT induces hepatic dysplasia. (Adobe pdf file .pdf)

Figure S5. Northern blot analysis showing that viral oncoprotein expression in livers of ApoE-rtTA:TRE2-TAg transgenic mice is not completely shut off in the absence of dox. (Adobe pdf file .pdf)

Figure S6. LT-dependent tumorigenesis is associated with altered hepatic differentiation and cell fate. IHC analysis for PCNA (proliferation), afp and biliary-specific cytokeratins (pan-CK). (Adobe pdf file .pdf)

Figure S7. Loss of PTEN accelerates 17kT-induced hyperplasia and LT-induced HCC by increasing hepatocyte proliferation, but not by further altering hepatic differentiation. (Adobe pdf file .pdf)

Figure S8. Immunoblot and Northern blot analysis showing that acceleration of LT-dependent HCC and 17kT-induced hyperplasia in the absence of PTEN is due to enhanced expression of the viral oncoproteins, rather than "super-activation" of PI3K and mTOR. (Adobe pdf file .pdf)

Supplementary Tables

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Supplementary Table S2: SV40-specific primers used for RT-PCR analysis and sequencing of viral transcripts (Adobe pdf file .pdf)

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Sheet 2: All mRNAs induced by 17kT and LT (≥ 3-fold up-regulated in 17kT and LT vs. WT)
Sheet 3: All mRNAs preferentially induced by LT (≥ 2-fold up-regulated in LT vs. 17kT)
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Supplementary Table S4: Table of down-regulated genes (Excel file .xls)
Sheet 1: All mRNAs down-regulated by 17kT or LT (≥ 3-fold down-regulated in either 17kT or LT vs. WT)
Sheet 2: All mRNAs down-regulated by 17kT and LT (≥ 3-fold down-regulated in 17kT and LT vs. WT)
Sheet 3: All mRNAs preferentially down-regulated by LT (≥ 3-fold down-regulated in LT vs. 17kT)
Sheet 4: All mRNAs preferentially down-regulated by 17kT (≥ 3-fold down-regulated 17kT vs. LT)

Supplementary Table S5: Sequence of primers used for semi-quantitative RT-PCR (Adobe pdf file .pdf)

Supplementary Table S6: Table of fetal hepatic stem cell-enriched mRNAs and their regulation by 17kT or LT (Adobe pdf file .pdf)

Supplementary Table S7: Table of mRNAs preferentially induced by 17kT: potential p53 targets or interactors (Adobe pdf file .pdf)

Supplementary Results

Livers of ApoE-rtTA:TRE2-TAg transgenic line 6-1 mice express 17kT and st, but not LT.

RT-PCR analysis demonstrated both qualitative and quantitative differences between viral transcripts expressed in both lines (Fig. S3 (a)). PCR with oligos SW9 and SW5 flanking the second LT intron amplified a 1044bp product in livers of mice from line 7-1 corresponding to the expected size for the normal LT and st-encoding viral pre-RNA, together with a product of ~300bp, the predicted size of the 17kT transcript (top panel, lane 3). This was in contrast to livers from 6-1 mice in which the ~300bp product was the only product amplified with SW9 and SW5, indicating that livers either failed to express the normal LT transcript or that splicing

preference favored the production of 17kT over LT (lane 2). This result was confirmed using oligos SW1 and SW5, a combination that amplified across the st intron, the common LT/17kT intron and the unique 17kT intron, which demonstrated amplification of 2 faint products of 1477bp and 1201bp and an abundant product of 455bp, corresponding to the predicted amplicons for the st, LT and 17kT transcripts respectively (lane 3), while the only product amplified in livers of line 6-1 was the 455bp 17kT transcript (lane 2). Immunoblotting with pab280 showed that livers of line 6-1 mice expressed a low level of st (Fig. 1b), a result that was at odds with our inability to detect length-full length st transcripts by RT-PCR with SW9/SW5 and SW1/SW5, combinations that had readily amplified LT and st transcripts in livers of 7-1 mice. Given that st protein is translated from a transcript with a long 3' untranslated region and SW5 is specific for nucleotides 3440-3421 within the C-terminus of the early region, the failure of this oligo combination to amplify LT or st specific transcripts in line 6-1, suggested that st protein was being translated from a truncated transcript in this line. To confirm that 6-1 livers expressed a transcript capable of producing full-length st protein, we performed RT-PCR using oligos SW1 and SW15. Oligo SW15 corresponds to nucleotides within LT and 17kT's common intron, thereby precluding the generation of RT-PCR products from LT or 17kT-specific transcripts when combined with any other oligo. However, when combined with SW1, SW15 should amplify an st-specific transcript of 314bp. RT-PCR with SW1 and SW15 amplified such a transcript in livers of PEPCK-TAg and ApoE-rtTA:TRE2-st transgenic livers, consistent with expression of st in both of these lines (Fig. 1b). In addition, SW1/SW15 amplified a 314bp product in ApoE-rtTA:TRE2-TAg lines 6-1 and 7-1 confirming that 6-1 livers did indeed express a transcript capable of producing st. Further confirmation of the specificity of SW1/SW15 for st transcripts was obtained by performing RT-PCR with this oligo combination on cDNA from livers of a line of ApoE-LT transgenic mice that express LT but not st (Comerford and Hammer, unpublished), which failed to amplify the 314bp product (data not shown). Thus, in livers of transgenic 6-1 mice, st is translated from a transcript that is either truncated or harbors deletions in its C-terminus, either of which would also preclude the production of full-length LT. Unambiguous identification of the abundant transcript in line 6-1 as *bona fide* 17kT, was obtained by direct sequencing of the 455bp PCR product amplified with SW1/SW5 and the 738bp product amplified with SW9/SW4 using sequencing primers SW18 and SW19 (Fig. S3 (b)). Sequencing of both products confirmed that the major transcript was 17kT, having arisen from excision of the common LT/17kT intron and the second 17kT intron using the splice donor site at nt 4425 and acceptor site at nt 3679 (Zerrahn et al., 1993). The 17kT transcript encoded a 135 amino acid protein that was co-linear with LT for the first 131 amino acids, with an additional 4 unique N-terminal amino acids (ALLT) followed by a stop codon.

17kT and LT both drive hepatocytes back into the cell cycle and induce hepatic hyperplasia, but only LT induces hepatic dysplasia.

To determine if the failure of 17kT to increase liver mass reflected an inability of 17kT to perturb hepatocyte proliferation or apoptosis, we placed mice on regular drinking water or water supplemented with 10μg/ml dox for 4 days or 4 weeks and determined % liver/body values and analyzed WT and transgenic liver histology by hematoxylin and eosin (H & E) staining and by immunohistochemistry (IHC) for proliferating cell nuclear antigen (PCNA)

(Fig. S4) and TUNEL (data not shown). In the absence of dox, hepatic architecture and hepatocyte morphology in livers of mice expressing 17kT were virtually indistinguishable from WT (Fig. S4A and B). However, while only 1 or 2 hepatocyte nuclei stained positive for PCNA in WT livers in any given microscopic field (Fig. S4D, arrowhead), 4-6 were commonly seen in 17kT livers (Fig. S4E, arrowheads), indicating that 17kT expression was not completely shut off in the absence of dox and that leaky 17kT expression was sufficient to drive a small number of hepatocytes back into the cell cycle. The suggestion that expression of LT was also leaky in livers of 7-1 mice was evident from the presence of larger-than-normal hepatocytes, many of which had massively enlarged nuclei that contained a larger number of extremely prominent nucleoli (Fig. S4C) and by the increased number of PCNA-positive hepatocytes (typically, 5-8/field) (Fig. S4F, arrowheads). Mitotic figures and TUNEL-positive hepatocytes were, however, absent indicating that the magnitude of expression of each transgene was not sufficient to induce widespread cell division or apoptosis. Northern blotting of liver RNA isolated from mice of both transgenic lines with and without dox-supplementation (Fig. S5) confirmed that expression of 17kT and LT was both leaky and dox-inducible.

The distinct effects of 17kT and LT on hepatocyte morphology became readily apparent when animals were provided with dox for just 4 days. While acute induction of 17kT promoted only a modest increase in cell and nuclear size, the presence of mitotic figures indicated that hepatocytes were now progressing through the cell cycle and actively dividing (Fig. S4H). In contrast, acute expression of LT induced severe hepatocyte dysplasia as seen by marked cell and nuclear hypertrophy, aberrant cell and nuclear morphology, infrequent mitoses, hyperchromasia and multinucleation (Fig. S4I). Despite these striking morphological differences between 17kT- and LT-expressing livers, however, there was no discernible difference in the number of PCNA-positive hepatocytes (Fig. S4K, L) or in the number of hepatocytes undergoing apoptosis (not shown) between each line. Thus while 17kT and LT both drove hepatocytes back into the cell cycle, 17kT promoted widespread cell division, while LT induced dysplasia and increased hepatocyte size by inducing endoreduplication and polyploidization.

Although 4 days of dox treatment failed to stimulate 17kT-dependent hepatomegaly, 4 weeks of dox treatment resulted in a doubling and tripling of liver mass in 17kT and LT livers respectively (Fig. 1a). In addition, the hepatocyte dysplasia observed after 4 days of LT induction (Fig. S4I) had, for the most part disappeared due to the replacement of dysplastic hepatocytes with small, highly proliferative mono-nucleated hepatocytes (Fig. S4O and R) similar to hepatocytes in 17kT-expressing livers (Fig. S4N and Q). Over time, sustained hepatocyte proliferation eventually resulted in hepatocyte crowding, sinusoidal compression and disruption of normal hepatic architecture such that normal liver zonation was no longer discernible in livers from either transgenic line.

LT alters hepatic differentiation and cell fate during tumorigenesis

Having shown that LT-induced hepatic dysplasia was associated with altered hepatic differentiation, we determined if altered differentiation also correlated with tumor development by performing IHC for PCNA and afp in livers of WT and transgenic mice provided with 25μg/ml dox for 6 weeks; a regimen that induced hyperplasia in 17kT livers and multi-focal tumor development in livers expressing LT (Fig. S6). Confirmation that cell fate and

differentiation was altered by LT was also obtained by performing IHC with a pan-cytokeratin-specific antibody (pan-CK) that recognizes a subset of cytokeratins that are expressed in hepatoblasts of the developing liver and which later become restricted to biliary epithelial cells (BECs) that line the bile ducts of the adult liver (C). IHC indicated that 17kT promoted sustained hepatocyte proliferation (D) without altering hepatic cell fate or differentiation as seen by the absence of afp-positive hepatocytes (E) and BEC-restricted pan-CK expression (F). In contrast, LT promoted the expansion of highly proliferative hepatic cells, most of which either expressed afp (H, K) or pan-CK (I, L) or, in rare instances, both (not shown). In some cases, nodules expressing higher levels of afp (dotted circle) could be seen within nodules expressing lower levels of afp (H) indicating that nodules contained hepatocytes with distinct differentiation potentials. Nodules demonstrating strong pan-CK staining were also evident in LT-expressing livers (I) as were clusters of hyperproliferating pan-CK-positive cells with ductal-like morphology (L).

LT-specific induction of afp and cd24a, together with the expression of biliary-specific cytokeratins in nodules also prompted us to compare the mRNAs induced by 17kT and LT with the broader list of known fhsc-associated mRNAs to determine the extent to which each viral oncoprotein impacted the expression of other fhsc markers. Supplementary Table S6 shows that of the 22 listed fhsc-associated mRNAs, 16 (72%) were induced by LT. Somewhat surprisingly, 13/22 (59%) were also induced by 17kT, although only 2 mRNAs (psat1 and uhrf1) demonstrated robust induction by 17kT relative to LT. Thus, LT's C-terminus perturbs the expression of developmentally regulated genes and genes within the imprinted network and rapidly shifts the hepatic transcriptome to a more fetal-like state.

PTEN-deficiency accelerates 17kT-induced hyperplasia and LT-dependent HCC by increasing hepatocyte proliferation and enhancing production of the viral oncoproteins.

To determine if loss of PTEN accelerated 17kT-induced hyperplasia or LT-dependent HCC by decreasing apoptosis, increasing proliferation or causing further alterations in hepatic differentiation, we performed TUNEL analysis and analyzed PCNA, afp and pan-CK expression by IHC in livers of mice of all genotypes after 4 weeks of dox treatment. TUNEL analysis showed that PTEN-deficiency had no effect on the rate of apoptosis in livers of either transgenic line (data not shown). However, Fig. S7 shows that while livers of WT mice (A), were devoid of PCNA- and afp-positive hepatocytes (B and C), and only demonstrated pan-CK immunostaining of BECs (D), livers lacking PTEN demonstrated focal areas of lipid accumulation (E) and a mild increase in the number of PCNA-positive hepatocytes (arrowheads, F), consistent with altered hepatocyte metabolism and a mildly elevated proliferation rate (Stiles et al., 2004). However, at this time, hepatocytes were negative for afp (G) and pan-CK expression remained confined to bile ducts (H), demonstrating that loss of PTEN *per se* did not alter hepatocyte differentiation.

Comparative analysis of 17kT livers with and without PTEN (I and M) showed that loss of PTEN increased hepatocyte number as seen by the increased density of small PCNA-positive hepatocytes per unit area (J and N), consistent with accelerated hyperplastic growth. While the absence of PTEN failed to alter afp (K, O) or CK (L, P) expression in 17kT-expressing hepatocytes, pan-CK+ cells (arrowheads, P) could sometimes be seen to necrotic areas (arrow, P) suggestive of hepatocyte crowding, sinusoidal compression and ongoing

liver repair. Interestingly, although loss of PTEN inhibits apoptosis by activating Akt (Downward, 2004), TUNEL analysis failed to demonstrate any significant difference between 17kT and Δ-PTEN/17kT livers with respect to hepatocyte apoptosis (data not shown) indicating that PTEN deficiency enhances 17kT-mediated liver growth by increasing hepatocyte proliferation rather than by inhibiting apoptosis or altering hepatocyte differentiation.

In contrast to the relatively mild differences between 17kT livers with and without PTEN, LT livers with and without PTEN differed dramatically after just 4 weeks on dox (Fig. S7, Q-X). In the presence of PTEN, livers expressing LT consisted of a generally homogeneous population of rapidly cycling PCNA-positive hepatocytes indicative of uniform hyperplastic growth (Q, R). PTEN deficiency, however, rapidly induced the growth of hepatic adenomas and multi-focal HCC (U) with variable proliferation rates (V), as well as tumors with ductal and cholangiocellular features (not shown). The difference between LT livers with and without PTEN was also reflected in the distribution of afp- (S, W) and CK- (T, X) positive cells. While afp-positive cells were restricted to small discrete nests dispersed throughout LT livers in the presence of PTEN (arrowheads, S), heterogeneous afp expression was now evident in adenomas and/or well- and poorly-differentiated HCCs in Δ-PTEN/LT livers (W). Similarly, CK immunoreactivity was restricted to BECs and the occasional solitary small cell within the parenchyma of PTEN-proficient LT livers (T), while Δ-PTEN/LT livers demonstrated intense CK staining in a subset of nodules (X). The overall rate of apoptosis was not significantly different between LT livers with and without PTEN, again indicating that loss of PTEN accelerateed LT-dependent tumor development primarily by increasing hepatocyte proliferation, rather than inhibiting apoptosis.

The DNA tumor virus oncoproteins activate PI3K/Akt and mTOR (Buchkovich et al., 2008), two signaling pathways that are negatively regulated by PTEN and constitutively activated in a variety of human cancers (Salmena et al., 2008). To determine if the failure of 17kT to induce tumors was due to an inability to activate signaling through either pathway, we immunoblotted liver extracts from WT and transgenic mice from both lines with phospho-specific antibodies to Akt and ribosomal protein S6 (S6), downstream effectors of PI3K and mTOR signaling, respectively (Fig. S8 (a)). Analysis showed that 17kT and LT modestly increased Akt phosphorylation without affecting total Akt levels. Both also induced S6 phosphorylation, the magnitude of which was consistently higher in LT livers. Immunoblotting with a PTEN-specific antibody confirmed that the viral proteins stimulated signaling to Akt and S6 without decreasing the levels of PTEN, demonstrating that the failure of 17kT to induce tumors was not due to an inability to activate PI3K/Akt or mTOR.

We next immunoblotted liver lysates from mice of all genotypes with Akt and S6-specific antibodies to determine if PTEN-deficiency enhanced 17kT-mediated hyperplasia or LT-dependent HCC by synergizing with the viral oncoproteins to “super-activate” Akt and mTOR. Antibodies specific for the viral oncoproteins and PTEN were also included to monitor for possible effects of viral protein expression on cre-mediated recombination of the $PTEN^{lox/lox}$ allele or to determine if viral protein expression was impacted by loss of PTEN. As shown in Fig. S8 (b), Akt and S6 were phosphorylated in WT and 7-1 livers lacking PTEN consistent with activation of PI3K and mTOR in response to loss of PTEN. Akt and S6 were also phosphorylated in 7-1 livers with PTEN, but only in the presence of dox, indicating that high levels of the viral oncoproteins were required to activate PI3K and mTOR. Importantly, in

transgenic livers lacking PTEN, the magnitude of phosphorylation was not significantly higher than in PTEN-proficient 7-1 livers indicating that the viral oncoproteins did not synergize with the absence of PTEN to hyperactivate PI3K or mTOR. Parallel analysis of signaling in 17kT livers with and without PTEN (Fig. S8 (c)) also failed to demonstrate synergy between 17kT and PTEN-deficiency for PI3K or mTOR activation. Thus PTEN deficiency does not enhance 17kT-mediated hyperplasia or LT-dependent HCC by hyperactivating PI3K or mTOR.

During the course of this analysis, we noticed that PTEN protein levels were higher in PTEN-deficient transgenic livers than in PTEN-deficient WT livers (compare PTEN levels in lanes 7 and 8, Fig. S8 (b) and lanes 3/4 and 7/8 Fig. S8 (c)), either suggesting that cre-mediated recombination was compromised by the extremely rapid rate of hepatocyte proliferation or that Alb-Cre transgene expression was reduced in the livers of 17kT and LT transgenic mice. Furthermore, hepatic expression of the viral oncoproteins was ~3-4-fold higher in mice lacking PTEN than in their PTEN-proficient counterparts (compare lanes 6 and 8, Fig. S8(b) and lanes 5/6 and 7/8 Fig. S8(c)). To determine if PTEN-deficiency increased viral oncoprotein abundance by increasing transcription of the viral RNAs, we performed Northern analysis of transgenic livers with and without PTEN (Fig. S8 (d)). Northern blotting confirmed that viral transcript abundance was not only higher in dox-treated mice expressing 17kT in the absence of PTEN (compare lanes 1 and 2), but was also higher in PTEN-deficient LT-expressing mice with and without dox treatment (compare lanes 3-6) indicating that PTEN-deficiency increased viral oncoprotein expression by increasing transcription and translation of the viral RNAs. Taken together, these results suggest that at least one mechanism by which PTEN-deficiency accelerates 17kT-mediated hyperplasia and LT-induced HCC is by increasing the dosage of the viral proteins, which in turn, enhances hepatocyte proliferation.

Supplementary Materials and Methods

Antibodies and Reagents for immunohistochemistry (IHC) and immunoblotting.

The following antibodies were obtained from Cell Signaling: S6 (#2212), Phospho-S6 (#2211), Akt (#9271), Phospho-Akt (#9271), Phospho-Akt (#9275) and PTEN (#9552). SV40 antibodies pab101 (#554149) and pab108 (#554150) were obtained from BD Biosciences, st-specific antibody pab280 (Ab-3) was from Calbiochem and UT450 was a gift from T.L Megraw (Department of Biomedical Sciences, Florida State University). The anti-β-tubulin antibody (T4026) was from Sigma. Afp (N1501) and pan-CK (Z0622) antibodies were from DAKO. p53 (NCL-p53-CM5p) and PCNA (NCL-L-PCNA) antibodies were from Novocastra. Donkey anti-mouse IgG-HRP (#715-035-150) was from Jackson Immunoresearch and donkey anti-rabbit IgG-HRP (NA934) was from Amersham Biosciences. Secondary antibodies for IHC were biotinylated goat-anti-rabbit IgG (DAKO #E0432) (for pan-CK, AFP or p53 IHC) or biotinylated rabbit-anti-mouse IgG (Jackson Immunoresearch, 315-065-003) (for SV40 LT or PCNA IHC). Streptavidin-conjugated HRP (43-4323) was from Zymed Laboratories.

Accompanying References for Supplementary Results and Supplementary Table S7

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