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Inhibition of U6 snRNA Transcription by PTEN

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Abstract: Problem statement: RNA polymerase III (RNA pol III) is responsible for transcribing many of the small structural RNA molecules involved in RNA processing and protein translation, thereby regulating the growth rate of a cell. RNA pol III transcribes both gene internal (tRNA) and gene external (U6 snRNA) promoters and proper initiation by RNA polymerase III requires the transcription initiation factor TFIIIB. TFIIIB has been shown to be a target of repression by tumor suppressors such as ARF, p53, RB and the RB-related pocket proteins. Also, TFIIIB activity is stimulated by the oncogenes c-Myc and the ERK mitogen-activated protein kinase. Recently, two TFIIIB subunits, BRF1 and BRF2, have been demonstrated to behave as oncogenes, making deregulation of TFIIIB activity and thus RNA pol III transcription an important step in tumor development. PTEN is a commonly mutated tumor suppressor regulating cell growth, proliferation and survival. Thus, we sought to examine the potential role of PTEN in regulating U6 snRNA transcription. **Approach:** We examined the potential for PTEN to regulate U6 snRNA transcription using *in vitro* RNA pol III luciferase assays, western blotting and deletion analysis in cancer cell lines differing in their PTEN status. **Results:** Using breast, cervical, prostate and glioblastoma cancer cells we demonstrate: (1) PTEN inhibition of gene external RNA pol III transcription is cell type specific, (2) PTEN-mediated inhibition of U6 transcription occurs via the C2 lipid-binding domain and (3) PTEN repression of U6 transcription occurs, at least in part, through the TFIIIB subunit BRF2. **Conclusion/Recommendations:** Our data demonstrates that regulation of the U6 snRNA gene by PTEN is mediated, in part by the TFIIIB oncogene BRF2, potentially identifying novel targets for chemotherapeutic drug design.

Key words: RNA polymerase, III transcription, PTEN, TFIIIB, BRF2

INTRODUCTION

RNA polymerase III (RNA pol III) transcribes genes involved in processing (U6 snRNA) and translation (tRNAs) and influences the growth rate of a cell (Schramm and Hernandez, 2002). RNA pol III recognizes different classes of promoters, which may be classified as gene internal, such as those found in the VAI and tRNA genes and gene external, as exemplified by the U6 snRNA gene (Schramm and Hernandez, 2002). TFIIIB is required for proper transcription initiation and in higher eukaryotes, at least two forms of TFIIIB have been identified (Schramm and Hernandez, 2002). Gene internal promoters, such as tRNA and VAI, require a form of TFIIIB consisting of TBP, Bdp1 and BRF1, whereas gene external promoters, such as U6 snRNA, require a TFIIIB complex containing TBP, Bdp1 and BRF2 (Schramm and Hernandez, 2002).

RNA pol III activity is sensitive to growth conditions and is tightly regulated throughout the cell cycle; RNA pol III activity is lowest during mitosis, then increases slowly through G1 and reaches its maximal activity during the S and G2 phases (White *et al*., 1995; Leresche *et al*., 1996). As such, RNA pol III transcription is regulated by tumor suppressors and oncogenes, most often by modulating TFIIIB activity (White, 2005). TFIIIB is specifically targeted by the tumor suppressors ARF (Morton *et al*., 2007), p53 (Felton-Edkins *et al*., 2003a), RB (Felton-Edkins *et al*., 2003a) and the RB-related pocket proteins p107 and p130 (Sutcliffe *et al*., 1999) and more recently BRCA1 (Veras *et al*., 2009). TFIIIB activity is stimulated by the

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oncogenes c-Myc (Felton-Edkins *et al*., 2003a), the casein kinase CK2 (Hu *et al*., 2004; Johnston *et al*., 2002) and the ERK (Felton-Edkins *et al*., 2003b) mitogen-activated protein kinase. RNA pol III transcription is also down-regulated by Maf1 during the growth cycle and in response to nutrient limitation, DNA damage, oxidative stress and a variety of drug treatments (Rollins *et al*., 2007; Johnson *et al*., 2007; Reina *et al*., 2006; Goodfellow *et al*., 2008). Maf1 represses RNA pol III transcription by targeting BRF2 (Rollins *et al*., 2007), BRF1 and RNA pol III directly (Reina *et al*., 2006). It has also been demonstrated that both gene internal (VAI) and gene external (U6 snRNA) RNA pol III transcription is regulated by chemopreventative agents such as EGCG (Jacob *et al*., 2007).

A definitive link between overexpression of RNA pol III product tRNAMet and transformation has been established (Marshall *et al*., 2008). Also, BRF2 expression and U6 snRNA gene external RNA pol III transcription correlate in all cancer cell lines tested implying a potential role for BRF2 as a regulator of cell proliferation (Cabarcas *et al*., 2008). More recently, BRF2 the TFIIIB subunit required for gene external RNA pol III transcription has been demonstrated to be over expressed in 40% of tumors from patients (>330) with non-small cell lung cancer and induced expression of BRF2 in bronchial epithelial cells made normal cells behave like cancer cells (Lockwood *et al*., 2010). Taken together, these data suggest that TFIIIB deregulation may be an important step in cancer development. Thus, increasing our understanding of how TFIIIB is regulated and deregulated, by tumor suppressors such as PTEN may lead to novel chemotherapeutic drug development, ultimately leading to better patient outcomes.

The phosphatase and tensin homolog deleted on chromosome ten (PTEN) is a tumor suppressor regulating cell growth, proliferation and survival found in almost all tissues. Somatic mutations in PTEN frequently occur in breast, prostate, ovarian, endometrial and brain tumors (Leslie and Downes, 2004; Waite and Eng, 2002; Simpson and Parsons, 2001). Germline mutations in PTEN result in autosomal dominant disorders such as Bannayan-Zonana syndrome and Cowden syndrome (Waite and Eng, 2002; Simpson and Parsons, 2001). Cowden syndrome is a familial cancer predisposition syndrome which is characterized by the development of multiple hamartomas in the breast and thyroid (Waite and Eng, 2002; Simpson and Parsons, 2001). Bannayan-Zonana syndrome, an autosomal dominant hamartomous disease, is characterized by macrocephaly, lipomatosis and hemangiomatosis (Waite and Eng, 2002; Simpson and Parsons, 2001). Additionally, the use of murine model to study the effects of PTEN deletion and mutation further supports the role of PTEN as a tumor suppressor. For example, mouse studies demonstrate that PTEN deficiency can result in abnormal development of germinal layers, spontaneous development of both thyroid and colon tumors and thymic lymphomas (Waite and Eng, 2002). Long-term follow up of these mice demonstrated there was development of both breast and endometrial cancers which are characteristic of Cowden syndrome (Waite and Eng, 2002). Although murine models never exhibit the exact characteristics seen in either Cowden or Bannayan-Zonana syndrome, the development of abnormalities are reminiscent of both syndromes (Waite and Eng, 2002) and it is clear that PTEN deficiency results in both diseases.

The PTEN structure includes a lipid phosphatase domain, a C2-lipid binding domain and a C-terminal tail (Lee *et al*., 1999). As an antagonist of the Phosphatidyl Inositol 3-Kinase (PI3K)/Akt pathway, PTEN controls cell growth and its phosphatase domain is essential for tumor suppression (Dillon *et al*., 2007). The C2-lipid binding domain regulates cell migration (Raftopoulou *et al*., 2003) and has anti-oncogenic function (Okumura *et al*., 2005). The C-terminal tail PEST and PDZ domains regulate PTEN stability (Georgescu *et al*., 1999).

PTEN has been shown to repress gene internal RNA pol III transcription by targeting the TFIIIB subunits TBP, BRF1 and Bdp1 thereby, interfering with proper assembly of TFIIIB (Woiwode *et al*., 2008). Furthermore, Woiwode *et al*. (2008) demonstrated that the ability of PTEN to function as a repressor of endogenous precursor tRNALeu transcription is independent of p53 and can be uncoupled from PTEN-mediated effects on the cell cycle.

PTEN also inhibits RNA pol I transcription by decreasing promoter occupancy, through TBP (Zhang *et al*., 2005). As BRF2 is also a subunit of TFIIIB (Schramm and Hernandez, 2002), we speculated that gene external RNA pol III transcription may be regulated by PTEN, through TFIIIB. Using breast, cervical, prostate and glioblastoma cancer cells we sought to determine if PTEN regulates U6 snRNA transcription and if PTEN regulates gene external RNA pol III transcription whether it occurs via the BRF2 subunit.

MATERIALS AND METHODS

Cell culture and luciferase assays: HeLa, DU-145, HCC1937, PC-3, MCF-7, MCF10A and U87 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cell media was supplemented with 5% FBS, L-glutamine, non-essential amino acids and penicillin/streptomycin (all supplies are from BioWhittaker, Walkersville, MD). The U6 and VAI luciferase vectors and transient transfections have been previously described (Rollins *et al*., 2007). Luciferase experiments were performed in triplicate, repeated three independent times and data presented are representative. Luciferase results are reported as Relative Light Units (RLU), representing the average of the *Photinus pyralis* firefly activity divided by the average of the *Renilla* luciferase vector. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (GraphpadPrism3.03, San Diego California, USA).

Mammalian expression constructs: pCDNA3-PTEN was generated as previously described (Bandyopadhyay *et al*., 2004). Flag tagged PTEN and PTEN gross deletions were constructed using PCR primers designed with Xba1 and BamH1 restriction sites, subsequently cloned into Xba1 and BamH1 sites of the mammalian expression vector p3XFlag-CMV-7.1 (Sigma). FlagBRF2 was cloned as previously described (Rollins *et al*., 2007). pSG5L-HAPTEN C124S (Plasmid 10744); pSG5L-HAPTEN G129E (Plasmid 10746); pSG5LFlagHAPTEN G129R (Plasmid 10775), were all obtained from Addgene (Cambridge, MA).

Western blot analysis: Nuclear extracts were prepared from HCC1937 cells transfected with an empty Flag vector or Flag-tagged PTEN and western blot was performed using anti-actin and anti-flag antibodies as previously described (Rollins *et al*., 2007). Cytoplasmic and nuclear extracts were prepared from HeLa cells transfected with Flag-tagged PTEN or Flag-tagged C2PTEN and western blot was performed using anti-flag antibodies as previously described (Rollins *et al*., 2007).

RESULTS

Inhibition of RNA pol III transcription by PTEN: To determine if PTEN can regulate gene external RNA pol III transcription, we monitored U6 snRNA transcription in a variety of cell lines differing in PTEN status. The HeLa cervical carcinoma cell line, DU-145 prostate carcinoma cell line, MCF-7 breast carcinoma cell line and MCF10A epithelial breast cell lines all express functional PTEN (Hlobilkova *et al*., 2000; Yu *et al*., 2002; McMenamin *et al*., 1999; Weng *et al*., 1999). We measured the effect of PTEN overexpression on both gene internal and external RNA pol III promoter activity in cells which express functional PTEN. HeLa, DU-145, MCF-7 and MCF10A cells were transfected with a promoterless pGL3 vector, pGL3-VAI (gene internal tRNA-like RNA pol III promoter) or pGL3-U6 (gene external RNA pol III promoter) and co-transfected with increasing concentrations of PTEN. Inhibition of VAI transcription by PTEN was statistically significant at the highest PTEN concentration tested in the HeLa cell line, $p<0.05$ (Fig. 1A). In the DU-145 cell line, VAI inhibition by PTEN was highly statistically significant at the two highest concentrations tested, $p<0.001$ (Fig. 1A). In the MCF-7 cell line, VAI inhibition by PTEN was statistically significant, $p<0.01$ and in the MCF10A cell line, VAI inhibition by PTEN was statistically significant, at all concentrations tested, $p<0.05$ and $p<0.001$, respectively (Fig. 1A). Strikingly, PTEN mediated inhibition of U6 snRNA transcription was highly statistically significant, $p<0.001$, in HeLa, DU-145, MCF-7 and MCF10A cells at almost all DNA concentrations tested (Fig. 1B). Interestingly, gene external RNA pol III transcription appears to be more sensitive to the effects of PTEN in HeLa, MCF-7 and MCF-10A cells.

Potentially the expression of both endogenous and exogenous PTEN together could influence the observed inhibition of both gene internal and external RNA pol III transcription. To rule out this possibility, we also measured the effect of PTEN overexpression on both gene internal and external RNA pol III promoter activity in the functionally null PTEN cells HCC1937, PC-3 and U-87. In the breast cancer HCC1937 cell line, VAI inhibition by PTEN was statistically significant, $p<0.01$, at the two highest concentrations tested (Fig. 2A). VAI inhibition by PTEN was statistically significant, $p<0.05$ and $p<0.01$, at the two highest concentrations tested (Fig. 2A) in the prostate cancer cell line PC-3. Lastly, in the glioblastoma U87 cell line, VAI inhibition by PTEN was significant, $p<0.01$ and $p<0.001$ respectively (Fig. 2A). These data are in agreement with a recent report demonstrating inhibition of VAI transcription by PTEN (Woiwode *et al*., 2008). Interestingly, Fig. 1A and 2A suggests that the magnitude of PTEN repression of gene internal RNA pol III transcription may be tissue specific.

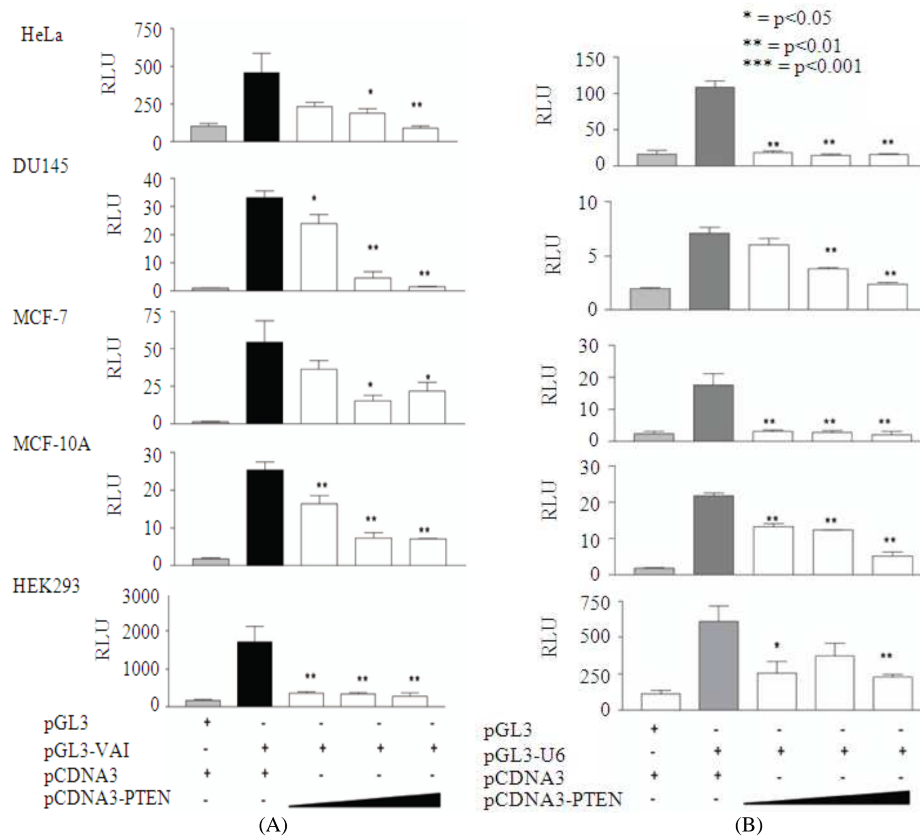

Fig. 1: PTEN inhibits RNA pol. III transcription in cancer cells which express functional PTEN. (A) HeLa, DU-145, MCF-7 and MCF10A cells transiently transfected with empty pGL3 vector (100 ng), pGL3-VAI (100 ng) alone or co-transfected with increasing concentrations of pCDNA3-PTEN (100, 125, 150 ng); (B) HeLa, DU-145, MCF-7 and MCF10A cells transiently transfected with empty pGL3 vector (100 ng), pGL3-U6 (100 ng) alone or cotransfected with increasing concentrations of pCDNA3-PTEN (50, 75, 100 ng). All luciferase assay results expressed as Relative Light Units (RLU): the average of the Photinus pyralis firefly activity observed divided by the average of the activity recorded from the Renilla luciferase vector. Experiments were done in triplicate, repeated three independent times and representative experiments are depicted. Statistical analysis was performed using oneway ANOVA with a Tukey post-test with a 95% confidence interval (Graphpad Prism 3.03); * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

Strikingly, PTEN mediated inhibition of U6 snRNA transcription in both PTEN null HCC1937 (breast) and PC-3 (prostate) cells was highly statistically significant (Fig. 2B). However, we did not see inhibition of U6 snRNA transcription by PTEN in PTEN null glioblastoma U87 cells (Fig. 2B). This data agrees with Woiwode *et al*. (2008) who did not see an effect on U6 transcription by PTEN in the U87 glioblastoma cell line. We cannot rule out the possibility that the effect of PTEN on U6 transcription may be cell type specific as we are able to show that PTEN significantly represses U6 transcription in a variety of cervical, prostate and breast carcinoma cell lines (Fig. 1B and 2B). Strikingly, for all breast and prostate cell lines tested, gene external RNA pol III transcription was more sensitive to inhibition by PTEN, as compared to gene internal RNA pol III transcription (Fig. 1 and 2).

To ensure that the observed repression of RNA pol III transcription was a result of exogenous PTEN expression, we prepared nuclear extracts from untransfected and transiently transfected HCC1937 cells with Flag-PTEN. Using an anti-flag antibody, we were able to detect PTEN in transiently transfected HCC1937 cells (Fig. 2C). The blots were probed with anti-actin as a loading control. Figure 2C confirms exogenous PTEN expression in the PTEN-null HCC1937 cells.

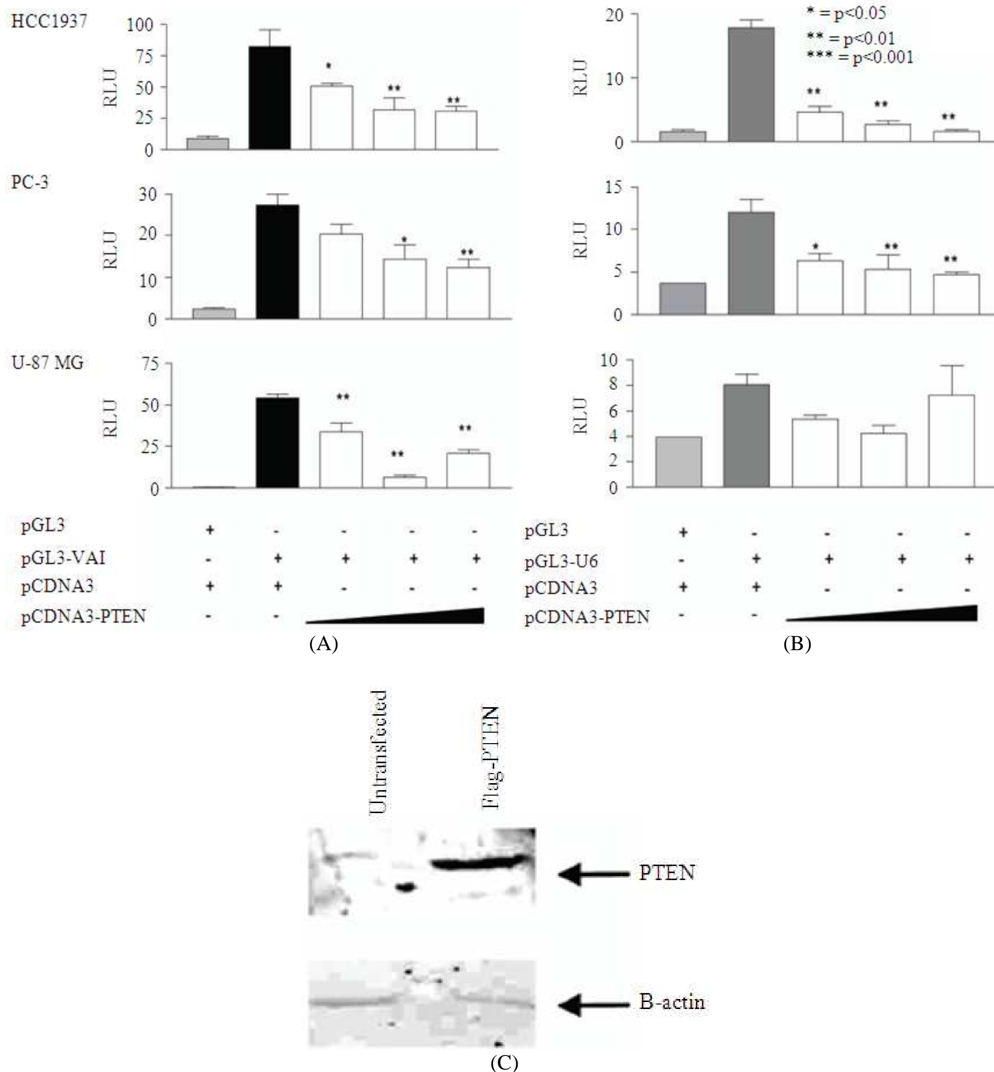

Fig. 2: PTEN inhibits RNA pol. III transcription in cancer cells which are PTEN-null. (A) PTEN-null HCC1937, PC-3 U87 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-VAI (100 ng) alone or cotransfected with increasing concentrations of pCDNA3-PTEN (100, 125, 150 ng); (B) PTEN-null HCC1937, PC-3 and U87 cells transiently transfected with empty pGL3 vector (100ng), pGL3-U6 (100 ng) alone or co-transfected with increasing concentrations of pCDNA3-PTEN (50, 75,100 ng); (C) Nuclear extracts prepared from untransfected HCC1937 cells or HCC1937 cells transiently transfected with Flag-PTEN and immunoblotted with anti-actin and anti-flag antibodies. All luciferase assay results expressed as Relative Light Units (RLU): the average of the Photinus pyralis firefly activity observed divided by the average of the activity recorded from the Renilla luciferase vector. Experiments were done in triplicate, repeated three independent times and representative experiments are depicted. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (Graphpad Prism 3.03); * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

To elucidate the mechanism (s) by which PTEN inhibits U6 snRNA transcription, we constructed gross PTEN deletions and tested their ability to inhibit gene internal and external RNA pol III transcription.

PTEN deletions inhibit U6 snRNA transcription: To determine the mechanism by which PTEN inhibits U6 transcription, we used previously characterized PTEN deletions (Odriozola *et al*., 2007), as depicted in Fig. 3A.

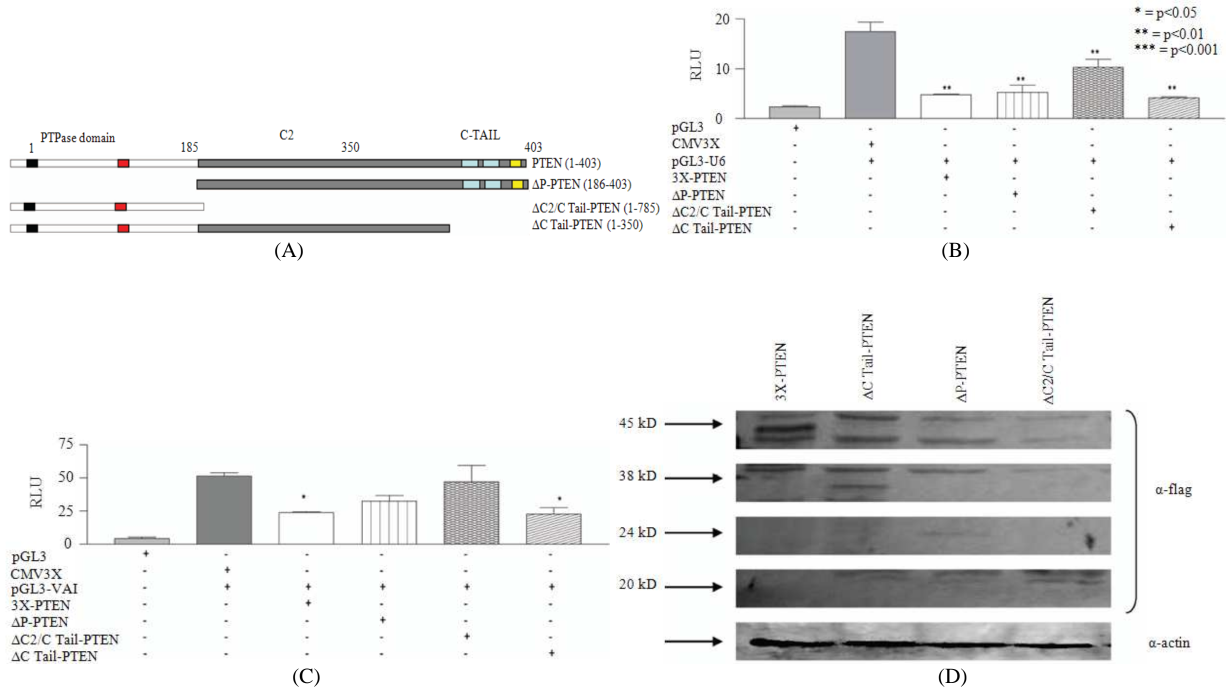

Fig. 3: U6 snRNA transcription is inhibited by PTEN deletions (A) Schematic representation of full-length PTEN and PTEN mutants**.** Full-length PTEN (1-403) represents the complete 403 amino acid protein. ΔP-PTEN (186-403) represents a deletion of the PTPase domain of PTEN. ΔC2/CTail-PTEN (1-185) represents a deletion of the C2 domain and the C-terminal tail. ΔCTail-PTEN (1-351) represents a deletion of the C-terminal tail. White box corresponds to the phosphatase domain which contains a black box corresponding to the PIP2-binding domain and a red box corresponding to the catalytic domain. Grey box corresponds to the C2-terminal half of PTEN containing the C2-lipid binding domain; blue boxes correspond to the PEST sequences and the yellow box corresponds to the PDZ binding domain; (B) PTEN-null HCC1937 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-U6 (100 ng) alone or co-transfected with 100 ng of the following: PTEN, ΔP-PTEN, ΔC2/CTail-PTEN and ΔCTail-PTEN; (C) PTEN-null HCC1937 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-VAI (100 ng) alone or co-transfected with 150 ng of the following: PTEN, ΔP-PTEN, ΔC2/CTail-PTEN and ΔCTail-PTEN. All luciferase assay results expressed as Relative Light Units (RLU): the average of the Photinus pyralis firefly activity observed divided by the average of the activity recorded from the Renilla luciferase vector. Experiments were done in triplicate, repeated three independent times and representative experiments are depicted. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (Graphpad Prism 3.03); * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

The PTEN deletions include: ΔP-PTEN, ΔC2/CTail-PTEN and ΔCTail-PTEN. ΔP-PTEN lacks the phosphatase domain and has been previously characterized for PTEN interaction (Odriozola *et al*., 2007) and membrane binding studies (Das *et al*., 2003). ΔC2/CTail-PTEN lacks the C2-lipid binding domain essential for proper positioning at the cell membrane and regulating cell migration (Raftopoulou *et al*., 2003) as well as the C-terminal tail containing PEST and PDZ domains responsible for PTEN stability (Georgescu *et al*., 1999). ΔCTail-PTEN lacks the C-terminal tail and has previously been used in cell membrane binding studies (Das *et al*., 2003).

HCC1937 cells were transiently transfected with pGL3-U6 or pGL3-VAI and with full-length PTEN or PTEN deletions. U6 snRNA transcription is highly statistically inhibited by the PTEN mutants ΔP-PTEN and ΔCTail-PTEN, $p<0.001$, Fig. 3B. ΔP-PTEN lacks the phosphatase domain and significantly inhibits U6 snRNA transcription, $p<0.001$, suggesting that the phosphatase domain may not be the primary PTEN domain responsible for gene external RNA pol III

inhibition. ΔCTail-PTEN, which has an intact C2 domain but lacks the C-terminal tail, also severely debilitates U6 snRNA transcription. Interestingly, U6 snRNA transcription is only slightly inhibited by PTEN deletion ΔC2/CTail-PTEN, which expresses the phosphatase domain alone $p<0.05$, Fig. 3B. Strikingly, U6 snRNA transcription is more sensitive to repression by PTEN mutants expressing the C2 domain, suggesting that the C2 domain may play a pivotal role in repressing gene external RNA pol III transcription.

To examine which portion of PTEN may be responsible for VAI inhibition, HCC1937 cells were transiently transfected with pGL3-VAI and with full length PTEN or PTEN deletions. VAI transcription is significantly inhibited by full-length PTEN and ΔCTail-PTEN, $p<0.05$ (Fig. 3C). The PTEN mutant ΔP-PTEN, lacking the phosphatase domain, is incapable of inhibiting VAI transcription (Fig. 3C) suggesting a role for the phosphatase domain in VAI mediated inhibition. This data is in agreement with a recent report demonstrating that the phosphatase domain is responsible for VAI inhibition (Woiwode *et al*., 2008). The ΔC2/CTail-PTEN mutant, lacking the C2 and C-tail domains, failed to inhibit VAI transcription. Thus, the possibility exists that the phosphatase domain itself may be unable to inhibit VAI transcription but other PTEN functional domains may be required to inhibit gene external RNA pol III transcription. Combined with the observation that deletion of the phosphatase domain did not affect PTEN's ability to inhibit U6 transcription (Fig. 3B) we further investigated the potential role of the PTEN phosphatase domain in regulating RNA pol III transcription, by testing several well characterized point mutations in the lipid phosphatase domain (Bandyopadhyay *et al*., 2004).

HCC1937 cells were transiently transfected with pGL3-U6 and with the well-characterized PTEN phosphatase domain mutants: G129E, G129R and C124S (Fig. 4A) (Ramaswamy *et al*., 1999). The PTEN phosphatase point mutations used in this study are depicted in Fig. 4. G129R is defective for both lipid binding and protein phosphatase activities. G129E, defective for lipid phosphatase activity, was previously used to demonstrate that PTEN mediated repression of gene internal RNA pol III transcription requires lipid phosphatase activities (Woiwode *et al*., 2008; Zhang *et al*., 2005). C124S, a phosphatase deficient and catalytically inactive mutant was tested as well (Ramaswamy *et al*., 1999). Figure 4A demonstrates that neither the lipid nor protein phosphatase activities are required for U6 inhibition by PTEN as G129E, G129R and C124S all significantly inhibit U6 activity, $p<0.001$, Fig. 4A. These data suggest that the PTEN phosphatase domain is not responsible for the observed gene external RNA pol III transcription inhibition by PTEN.

In the case of VAI transcription, Fig. 3B suggested that the phosphatase domain may be responsible for the observed inhibition of gene internal RNA pol III transcription. To further examine the role of the PTEN phosphatase domain in regulating gene internal RNA pol III transcription, we transiently transfected HCC1937 cells with pGL3-VAI and the well characterized phosphatase domain mutants, G129E, G129R and C124S, Fig. 4B. Figure 4B demonstrates that none of the phosphatase point mutations tested are capable of inhibiting VAI activity. These data suggest that mutation or complete deletion of the phosphatase domain compromises the ability of PTEN to inhibit gene internal RNA pol III transcription, Fig. 3B and 4B. This data is in agreement with the data presented by Woiwode *et al*. (2008) who demonstrated a role for the phosphatase domain of PTEN in inhibiting gene internal RNA pol III transcription.

These data suggest that the phosphatase activities of PTEN are responsible for VAI inhibition but do not play a role in inhibition of gene external U6 snRNA transcription, Fig. 3-4. Interestingly, in the case of U6 snRNA transcription, the ΔC2/CTail-PTEN mutant inhibits, $p<0.05$, much less than full-length PTEN, ΔP-PTEN and ΔCTail-PTEN ($p<0.001$) (Fig. 3A). This suggests the C2 domain of PTEN may be important in regulating gene external RNA pol III transcription. To test this hypothesis, we generated a previously characterized mutant expressing the PTEN C2 domain alone (Chu *et al*., 1997) and tested its ability to affect RNA pol III transcription.

The C2 domain is responsible for PTEN inhibition of U6 snRNA transcription: The C2 domain can regulate cell migration (Raftopoulou *et al*., 2003) and has anti-oncogenic function independent of the phosphatase activity (Okumura *et al*., 2005). To determine if the C2 domain alone could inhibit U6 snRNA transcription we constructed a previously characterized C2 domain construct (Fig. 5) (Raftopoulou *et al*., 2003; Das *et al*., 2003). HCC1937 cells were transiently transfected with pGL3-U6 and full-length PTEN or pGL3-U6 and C2-PTEN. Strikingly, the C2 domain alone is capable of significantly inhibiting U6 promoter activity, $p<0.001$ (Fig. 5A).

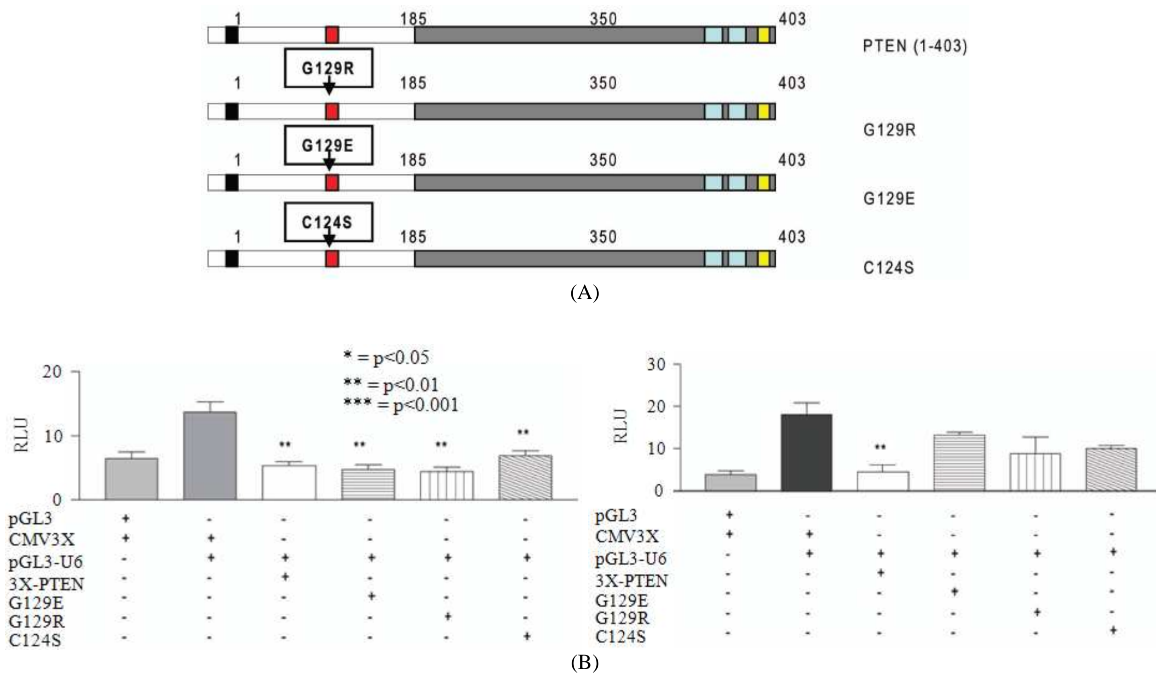

Fig. 4: PTEN phosphatase point mutations inhibit U6 snRNA transcription. Schematic of well-characterized PTEN phosphatase point mutations in the catalytic site. (A) PTEN-null HCC1937 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-U6 (100 ng) alone or co-transfected with 100 ng of the following: following: pSG5L HA PTEN G129E; pSG5L Flag HA PTEN G129R; pSG5L HA PTEN C124S; (B) PTEN-null HCC1937 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-VAI (100 ng) alone or co-transfected with 150 ng of the following: following: pSG5L HA PTEN G129E; pSG5L Flag HA PTEN G129R; pSG5L HA PTEN C124S. All luciferase assay results expressed as Relative Light Units (RLU): the average of the Photinus pyralis firefly activity observed divided by the average of the activity recorded from the Renilla luciferase vector. Experiments were done in triplicate, repeated three independent times and representative experiments are depicted. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (Graphpad Prism 3.03); * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

We further tested if the C2 domain plays a role in PTEN mediated inhibition of VAI activity, we transiently transfected HCC1937 cells with pGL3-VAI and full-length PTEN or pGL3-VAI and C2-PTEN. Figure 5B demonstrates the C2-PTEN construct is not capable of inhibiting VAI activity, further confirming the role of the PTEN phosphatase domain in regulating gene internal RNA pol III transcription. The observation that the C2-PTEN mutant preferentially inhibits U6 snRNA transcription, but not VAI, suggests that the mutant is indeed being expressed (Fig. 5A-B). However, to confirm the expression and localization of the C2-PTEN mutant, nuclear and cytoplasmic extracts from HeLa cells transiently transfected with Flag alone or FlagC2PTEN were prepared and western blotting confirmed that the C2 mutant localized to the cell nucleus (Fig. 5C).

How PTEN enters the nucleus appears to be influenced by a variety of mechanisms (Planchon *et al*., 2008). NLS-like sequences within PTEN and amino acids 265-269, which are within the C2 domain, were shown to be necessary for nuclear import (Chung *et al*., 2005). Also, the C2 domain has been shown to interact with the Major Vault Protein (MVP) in HeLa cells providing a mechanism by which PTEN can enter the nucleus (Yu *et al*., 2002). MVP is one of three proteins which comprise mammalian vaults, a ribonucleoprotein particle which is proposed to function as a nuclear-cytoplasmic transporter (Mossink *et al*., 2003). Thus, we speculate that the C2 domain of PTEN may be capable of repressing RNA pol III transcription by entering the nucleus via the MVP or the NLS-like sequence within amino acids 265-269.

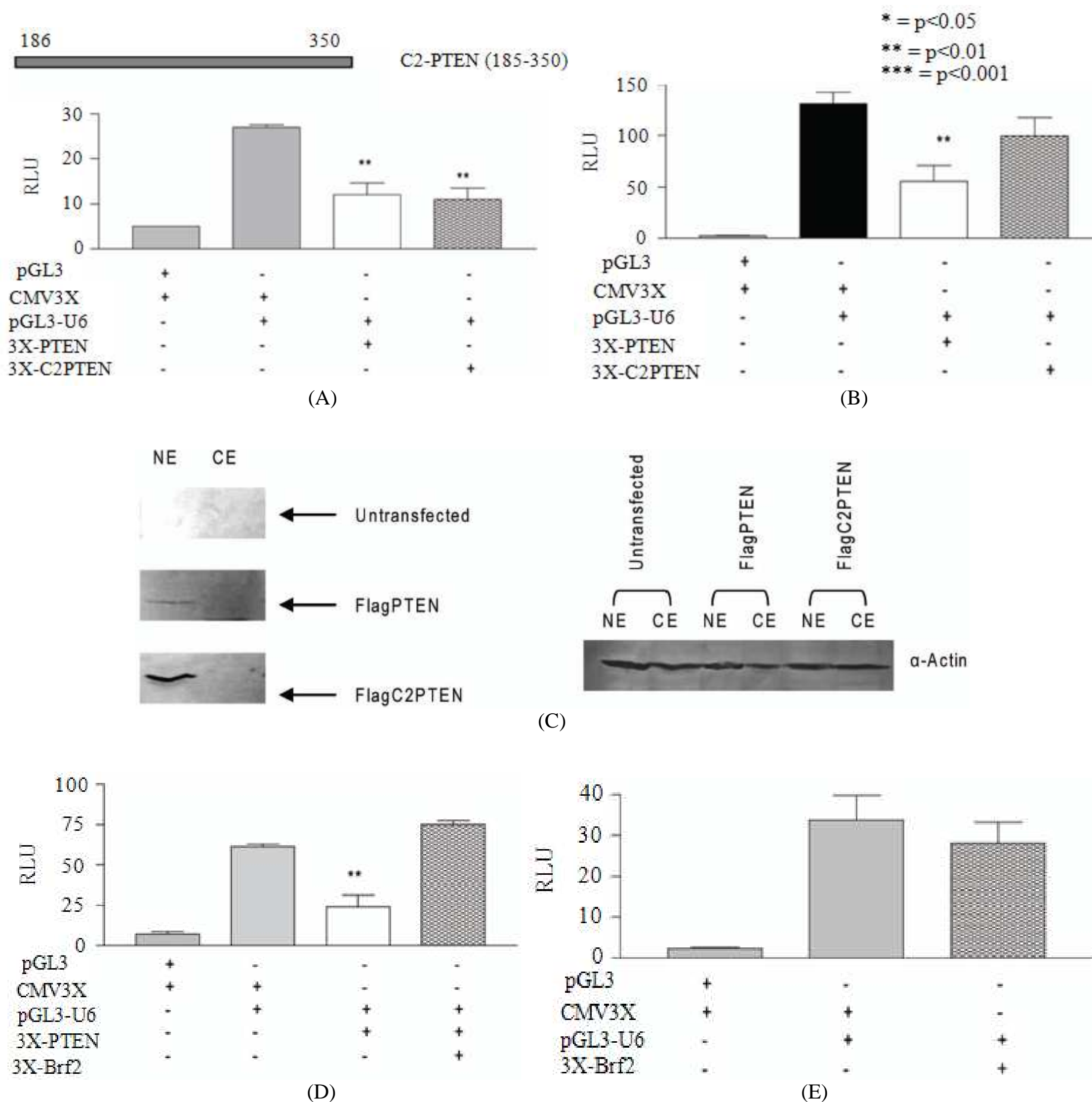

Fig. 5: The C2 domain is responsible for PTEN inhibition of U6 snRNA transcription (A) Schematic of C2-PTEN domain alone. PTEN-null HCC1937 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-U6 (100 ng) alone or co-transfected with 100 ng of full-length PTEN or C2-PTEN; (B) PTEN-null HCC1937 cells transiently transfected with empty pGL3 vector (100 ng), pGL3-VAI (100 ng) alone or co-transfected with 150 ng of full-length PTEN or C2-PTEN; (C) Nuclear and cytoplasmic extracts prepared from untransfected HeLa cells or HeLa cells transiently transfected with Flag-PTEN and Flag-C2PTEN and immunoblotted with anti-actin and ntiflag antibodies; (D) PTEN-null HCC1937 cells transiently transfected with pGL3 (100 ng), pGL3-U6 (100 ng) alone, pGL3-U6 (100 ng) and full-length PTEN, or co-transfected with pGL3-U6, full-length PTEN and BRF2; (E) PTENnull HCC1937 cells transiently transfected with pGL3 (100 ng), pGL3-U6 (100ng) alone, or co-transfected with pGL3-U6 and BRF2. All luciferase assay results expressed as Relative Light Units (RLU): the average of the 10 Photinus pyralis firefly activity observed divided by the average of the activity recorded from the Renilla luciferase vector. Experiments were done in triplicate, repeated three independent times and representative experiments are depicted. Statistical analysis was performed using one-way ANOVA with a Tukey post-test with a 95% confidence interval (Graphpad Prism 3.03); * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$

The data presented suggests a role for the C2 domain in PTEN-mediated regulation of gene external RNA pol III transcription. We demonstrate that the C2 domain itself is capable of regulating U6 snRNA transcription (Fig. 5A). The precise mechanism by which PTEN inhibits gene external RNA pol III transcription has yet to be elucidated, but the observed inhibition of U6 and VAI transcription by PTEN was not equivalent (Fig. 1 and 2), suggesting that the RNA pol III enzyme itself may not be the primary target. Thus, we speculate that regulation of gene external RNA pol III transcription by PTEN may involve TFIIIB.

PTEN repression of U6 snRNA transcription occurs via the TFIIIB subunit BRF2: To determine if PTEN inhibits U6 snRNA transcription through TFIIIB, we overexpressed the TFIIIB subunit BRF2 in HCC1937 cells. HCC1937 cells were transiently transfected with pGL3-U6, pGL3-U6 and PTEN, or pGL3-U6, PTEN and BRF2. Interestingly, overexpression of the TFIIIB subunit BRF2 (Fig. 5D) is capable of alleviating PTEN mediated repression of U6 snRNA transcription and restoring transcription to levels of pGL3-U6 alone. To ensure that BRF2 was not limiting in our assay, we transiently transfected HCC1937 cells with pGL3-U6 alone and co-transfected with pGL3-U6 and BRF2 (Fig. 5E). As demonstrated, co-transfecting BRF2 with pGL3-U6 does not increase activity of U6 (Fig. 5E). These data suggest that PTEN exerts its repressional activity, at least in part, through the TFIIIB subunit BRF2, recently demonstrated to be an oncogene (Lockwood *et al*., 2010).

DISCUSSION

Here we report the inhibition of U6 snRNA transcription by PTEN in cervical, prostate and breast cancer cell lines. We also show that in breast and prostate cancer cells U6 snRNA transcription is more sensitive to PTEN mediated inhibition than VAI transcription (Fig. 1 and 2). Using previously characterized PTEN domain deletions and phosphatase point mutations, we demonstrate that the phosphatase domain is responsible for repression of VAI promoter activity (Fig. 3B and 4B) and further demonstrate that the C2 domain can independently repress U6 promoter activity (Fig. 3A and 5A). PTEN has been demonstrated to inhibit cell migration through its C2 domain, independent of its lipid phosphatase activity and that the control of cell migration through its C2 domain is expected to be an important feature of its tumor suppressor activity (Raftopoulou *et al*., 2003; Freeman *et al*., 2003). Additionally, Woiwode *et al*. (2008) demonstrated that the ability of PTEN to inhibit tRNALeu transcription is independent of p53, we speculate that PTEN-mediated inhibition of U6 snRNA transcription in HCC1937 cells may also be independent of p53 as HCC1937 cells are p53 null (DelloRusso *et al*., 2007).

This report demonstrates a novel role for the C2 domain of PTEN, independent of its phosphatase activity, as a regulator of U6 transcription. We further demonstrate that overexpression of the TFIIIB subunit BRF2 can alleviate PTEN inhibition of U6 snRNA transcription (Fig. 5D). As both gene internal and external TFIIIB complexes share TBP and Bdp1, it is possible PTEN may regulate U6 snRNA transcription via TBP and Bdp1 as well and remains to be tested.

We speculate that the observed PTEN tissue specific repression of gene internal and gene external RNA pol III transcription occurs via two independent mechanisms. In the case of VAI transcription, the lipid phosphatase domain of PTEN is required (Fig. 3D and 4C) and inhibition occurs via the TFIIIB subunits BRF1 and Bdp1 (Woiwode *et al*., 2008). Inhibition of U6 snRNA transcription requires the C2 domain of PTEN (Fig. 3B and 5A) and occurs via the TFIIIB subunit BRF2 (Fig. 5D).

CONCLUSION

This report, to the best of our knowledge, provides the first evidence demonstrating tissue specific regulation of U6 snRNA transcription by PTEN, via the TFIIIB oncogene BRF2. We also demonstrate a novel role for the C2 domain of PTEN in the regulation of U6 snRNA transcription. Together, these data provide evidence for the rationale design of novel cancer therapeutics.

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