TUBB3/βIII-tubulin acts through the PTEN/AKT signaling axis to promote tumorigenesis and anoikis resistance in non-small cell lung cancer.

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PRECISE: Findings revealed how a structural protein tightly associated with aggressive disease and therapeutic resistance in lung adenocarcinomas and other cancers influences tumor growth.
ABSTRACT

βIII-tubulin (TUBB3) expression is associated with therapeutic resistance and aggressive disease in non-small cell lung cancer (NSCLC), but the basis for its pathogenic influence is not understood. Functional and differential proteomics revealed that βIII-tubulin regulates expression of proteins associated with malignant growth and metastases. In particular, the adhesion-associated tumor suppressor maspin was differentially regulated by βIII-tubulin. Functionally, βIII-tubulin suppression altered cell morphology, reduced tumor spheroid outgrowth and increased sensitivity to anoikis. Mechanistically, the PTEN/AKT signaling axis was defined as a critical pathway regulated by βIII-tubulin in NSCLC cells. βIII-tubulin blockage in vivo reduced tumor incidence and growth. Overall, our findings revealed how βIII-tubulin influences tumor growth in NSCLC, defining new biological functions and mechanism of action of βIII-tubulin in tumorigenesis.
INTRODUCTION
Lung cancer is a lethal adult cancer accounting for the most cancer deaths worldwide (1). The most common form, Non-Small Cell Lung Carcinoma (NSCLC) represents >80% of all cases (2). Over half of NSCLC patients have developed metastasis at the time of diagnosis and 5-year survival is ~14% (2). Advanced NSCLC is poorly responsive to therapy and the mechanisms responsible for its resistance and aggressive behavior are not well defined.

Alterations in expression of microtubule proteins in cancer cells are emerging as important contributors to chemotherapy resistance (3, 4). Microtubules are cytoskeletal proteins that comprise α- and β-tubulin heterodimers and are involved in many important cellular processes including maintenance of shape, intracellular transport and mitosis [reviewed in (4)]. These critical roles make microtubules attractive drug targets for anticancer therapies. Indeed, chemotherapy agents including the microtubule stabilising (e.g. taxanes) and destabilising (e.g. vinca alkaloids) drugs exert their toxic effect by binding the β-tubulin subunit of microtubules to induce a potent mitotic block [reviewed in (4)]. β-tubulin has seven isotypes that display tissue-specific expression. For example, βI-tubulin is constitutively expressed in many tissues, whereas βII-tubulin and βIII-tubulin are expressed in neuronal tissues, and βIV-tubulin is restricted to hematopoietic tissues (3, 4). Clinical studies show that high βIII-tubulin expression correlates with chemoresistance and poor survival in different tumor types, including breast, ovarian, gastric and NSCLC (5, 6). Previously we demonstrated a functional role for βIII-tubulin in regulating chemosensitivity in NSCLC using RNA interference to silence βIII-tubulin expression in NSCLC cells (7-10). Knockdown of βIII-tubulin
increased sensitivity via an increase in apoptosis to chemotherapeutic agents both in vitro and in vivo (7-10). However, despite evidence to link βIII-tubulin expression to chemotherapy drug sensitivity, its roles in the tumorigenic and metastatic potential of NSCLC are unknown. Recent clinical data has suggested that high βIII-tubulin expression is correlated to aggressive and metastatic tumors (11, 12). In addition, we recently showed that silencing βIII-tubulin expression in the absence of chemotherapy delayed tumor growth in a mouse model of lung cancer (10). However, how βIII-tubulin exerts its effect on tumor growth and its role in metastases of tumor cells remain unknown.

In this study, we show for the first time that stable suppression of βIII-tubulin in NSCLC cells alters the expression of key proteins involved in regulating tumorigenic and metastatic potential, alters cell morphology, increases anoikis sensitivity, and modulates PTEN/AKT signaling. Finally, we demonstrate that knockdown of βIII-tubulin in NSCLC cells decreases the incidence and growth of lung tumors in two different preclinical mouse models.
METHODS

Cell culture.

H460 and A549 NSCLC cells were obtained from ATTC and grown as described (8-10). H460 (pRS/βIII_SH4 and pRS/βIII_SH59) and A549 (pRS/βIII_SH61) cell clones stably expressing shRNA targeting βIII-tubulin or control (non-functional) shRNA (pRS/Ctrl_SH1, pRS/Ctrl_SH2, pRS/Ctrl_SH27), and two H460 βIII-tubulin ‘rescue’ cell clones (pRS/βIII_SH4/R6 and pRS/βIII_SH4/R17) were developed and characterized as described (10). All cells were routinely screened and found to be free of mycoplasma.

Two-Dimensional Differential Gel Electrophoresis (2D-DIGE).

2-D DIGE is a fluorescent based proteomics approach that uses charge and size-matched CyDye fluorophores that covalently attach to lysine residues for protein labeling prior to fractionation. A key strength of this assay is the ability to directly compare protein samples using a multiplex approach, while incorporating a standardized internal control (13, 14). To identify differentially expressed proteins in NSCLC cells with stable suppression of βIII-tubulin, we performed 2-D DIGE on H460 βIII-tubulin shRNA (pRS/βIII_SH4 and pRS/βIII_SH59) or control shRNA (pRS/Ctrl_SH1 and pRS/Ctrl_SH2) cells (Supplementary Fig 1). 2D-DIGE was performed as we previously described with modifications (15). Refer to Supplementary Method for detailed methodology. For analysis of the effects of βIII-tubulin knockdown, H460 pRS/βIII_SH4 and pRS/βIII_SH59 cells were grouped and compared to controls, H460 pRS/Ctrl_SH1 and pRS/Ctrl_SH2 cells. These comparisons were performed for both nuclear and cytoplasmic fractions at two pH ranges, pH 4.5-5.5 (narrow) or pH 4-7 (broad). Analysis of the fluorescent gel images was performed using the Batch Processor and Biological
Variation Analysis (BVA) Module of the DeCyder software version 5.0. The BVA module was used to compare each group and data is expressed as average expression ratios and student’s t-tests of individual protein spots.

**MALDI-TOF mass spectrometry.**

Protein spots were excised from SYPRO Ruby stained gels and MALDI-TOF mass spectrometry performed as described (15). Peptide masses were searched against SwissProt/TREMBL protein databases using the PeptIdent tool on ExPASy (http://tw.expasy.org/tools/peptident.html) for protein identification.

**Western blotting.**

Western blot analysis was performed using the following antibodies; βIII-tubulin (clone TUJ1, Chemicon), maspin (BD Biosciences), profilin 2 (AbCam), peroxiredoxin 4 (AbCam), total β-tubulin (AbCam), lamin B1 (AbCam), p-AKT (S473), pAKT (T308), total AKT, PTEN (Cell Signaling Technology) and GAPDH (Abcam) as described (10, 16).

**Immunofluorescence microscopy.**

The actin cytoskeleton of NSCLC cells was visualized by phalloidin staining and imaged using a Zeiss immunofluorescence microscope (Zeiss).

**Real time quantitative PCR (qPCR) analysis.**

The expression of βIII-tubulin and maspin mRNA in NSCLC cells was examined by qPCR as described (10, 16). All data were normalized to the housekeeping gene β2-microglobulin.
Spheroid outgrowth assay

Tumor multicellular spheroids were generated in 96-well round bottom plates as described (17). After formation, spheroids of uniform size were transferred to 6-well tissue culture plates and left to attach and grow for 24h. Images were captured on a Zeiss Axiovert 200M inverted microscope and cell outgrowth quantified using the AxioVision software package.

Anoikis assay.

To measure anchorage-independent cell death, βIII-tubulin shRNA expressing (pRS/βIII_{SH4} and pRS/βIII_{SH59}), control (pRS/Ctrl_{SH1} and pRS/Ctrl_{SH2}) or βIII-tubulin rescue (pRS/βIII_{SH4}/R17) cells were seeded onto poly-(2-hydroxyethyl methacrylate)-coated tissue culture plates. 48h post-seeding cells, cell death was measured as described (10, 18).

In vivo mouse models.

Experimental Metastasis Model. All animal experiments were approved by the Animal Ethics committee, UNSW (ACEC no. 12/41A). Balb/c nude mice received a tail vein injection of 5x10^5 cells stably expressing βIII-tubulin (pRS/βIII_{SH4} and pRS/βIII_{SH59}) or control (pRS/Ctrl_{SH2} and pRS/Ctrl_{SH1}) shRNA. The detection of tumors within the lung was monitored by micro-CT imaging.

Tumorigenicity Model. BALB/c nude mice were inoculated subcutaneously into the flank with 1x10^6 NSCLC cells expressing βIII-tubulin (pRS/βIII_{SH4} and pRS/βIII_{SH59}) or control (pRS/Ctrl_{SH1} and pRS/Ctrl_{SH2}) shRNA resuspended in PBS to mimic anchorage-
independence or in matrigel to mimic anchorage-dependence as described (10, 19). Tumors were measured as described (10, 19).

**Micro-CT tumor analysis.**

The presence of lung tumors in the mice was monitored by micro-computed tomography (CT) scanning. The micro-CT data were acquired using an Inveon system (Siemens) at 71.88 pixel size, 220 projections, 250ms integration time, 50keV photon energy and 450µA current. Image data were evaluated using the Inveon software package (Siemens). Tumor margins were identified by contrast thresholding which allowed the tumor margins (soft tissue density) to be defined against the surrounding lung (air density).

**STATISTICS**

Data are expressed as the mean ± SE and analyzed using ANOVA or Student's t test followed by the nonparametric Dunnett test using the GraphPad Prism program. A P value of <0.05 was considered statistically significant.
RESULTS

Suppression of βIII-tubulin alters the expression of proteins involved in regulating tumorigenesis and metastases.

Previously, we established NSCLC cells which have stable and potent suppression of βIII-tubulin (10). These cells displayed increased chemosensitivity and decreased tumour incidence (10). However, despite clinical reports correlating increased levels of βIII-tubulin in NSCLC with aggressive disease, studies describing its role in tumorigenicity and metastases are limited. To investigate the role of βIII-tubulin 2D-DIGE proteomics was performed on both the cytoplasmic and nuclear fractions of 2 independent TUBB3/βIII-tubulin shRNA expressing H460 NSCLC cell clones (pRS/βIII SH4 and pRS/βIII SH59) and 2 independent control shRNA H460 NSCLC cell clones (pRS/Ctrl SH1 and pRS/Ctrl SH2 (Supplementary Figures 1 and 2). Differences in protein expression between the βIII-tubulin shRNA expressing and control cells were quantitatively determined using the DeCyder image analysis software, which provided statistical information on each individual protein identified on the 2D-DIGE gel. Protein spots of interest were excised and identified by mass spectrometry. Eleven out of a total of 963 proteins (1.1%; pI 4-7, broad range) and 56 (21 up regulated and 35 down regulated) out of a total of 753 proteins (7%; pI 4.5-5.5, narrow range) were significantly altered in the cytoplasmic fractions of H460 pRS/βIII SH4 and SH59 cells compared to controls (pRS/Ctrl SH1 and SH2). Moreover, 33 out of a total of 1331 proteins (2.5%; pI 4-7, broad range) and 42 (9 up regulated and 33 down regulated) out of a total of 502 proteins (8.4%; pI 4.5-5.5, narrow range) were significantly altered in the nuclear fractions of pRS/βIII SH4 and SH59 cells compared to controls. A number of proteins
identified as being differentially expressed in the βIII-tubulin knockdown cells, are involved in modulating tumor biology (Supplementary Tables 1 and 2). To confirm changes in protein expression and mass spectrometry analysis, a select number of proteins were validated by western blotting as being differentially expressed in both individual βIII-tubulin shRNA cell clones (pRS/βIII_SH4 and SH59) compared to controls (pRS/Ctrl_SH1 and SH2) (Supplementary Figure 4).

**βIII-tubulin levels influence the expression of the tumor suppressor maspin in NSCLC cells.**

Maspin also known as Serpin B5 is a member of the serine protease inhibitor/non-inhibitor superfamily [reviewed in (20)] and is classified as a tumor suppressor in different cancer types (20). Maspin was identified by 2D-DIGE as being upregulated (2.29 fold, p<0.05) in the βIII-tubulin shRNA expressing cells (Supplementary Table 1). Western blot analysis confirmed the increase in maspin protein levels in pRS/βIII_SH4 and SH59 cells when compared to controls (Figure 1A-B). Recently, studies have described the localization of maspin in the cytoplasm or the nucleus as being an important determinant in its tumor suppressor activity (21, 22). To examine whether there was any difference in the cellular distribution of maspin in βIII-tubulin shRNA expressing cells, cytoplasmic and nuclear extracts were collected and maspin expression measured by western blot. Control shRNA expressing cells had higher maspin expression in their cytoplasm when compared to the nucleus (Figure 1C). βIII-tubulin shRNA expressing cells also had increased maspin expression in their cytoplasmic fraction compared to the nuclear fraction (Figure 1C). However, the expression of maspin was increased in both the cytoplasmic and nuclear fractions of the βIII-tubulin shRNA cells compared to
control cells (Figure 1C). To determine whether this difference was reflected at the gene level, maspin gene expression was measured by qPCR. Maspin mRNA levels were increased in both βIII-tubulin shRNA expressing cell clones (pRS/βIII\textsubscript{SH4} and \textsubscript{SH59}) compared to controls (pRS/Ctrl\textsubscript{SH1} and \textsubscript{SH2}) (Figure 1D). The increase in maspin expression was also confirmed in another H460 NSCLC βIII-tubulin shRNA independent cell clone (pRS/βIII\textsubscript{SH60}) which also displayed potent knockdown of βIII-tubulin (Supplementary Figure 5). Notably, this cell clone was not used in the 2D-DIGE experiments, thus adding further support to the correlation between βIII-tubulin knockdown and increased maspin expression.

To determine whether βIII-tubulin expression can directly influence maspin expression, βIII-tubulin was restored back into the βIII-tubulin shRNA expressing cells as described (10) and maspin expression measured. Restoration of βIII-tubulin expression in 2 individual βIII-tubulin rescue clones (pRS/βIII\textsubscript{SH4}/R6 and pRS/βIII\textsubscript{SH4}/R17) completely abolished the increased expression of maspin (Figure 2A-C). In contrast, the empty pREP vector clone (pRS/\textsubscript{EV}) which maintained suppressed βIII-tubulin expression (Figure 2A) did not alter maspin expression (Figure 2A-C). Collectively, these results show for the first time that βIII-tubulin levels directly influence the expression of a tumor suppressor protein.

**βIII-tubulin suppression induces cell morphology changes in NSCLC cells.**

Malignant transformation of tumor cells is associated with changes in organization of the cell cytoskeleton, decreased cell adhesion, increased cell migration and resistance to anchorage-independent apoptosis (23). Given that suppression of βIII-tubulin led to alterations in the expression of proteins involved in regulating tumor growth and
metastases, we assessed whether suppression of βIII-tubulin led to changes in cell morphology of NSCLC cells. βIII-tubulin knockdown produced marked changes in cellular morphology when compared to controls. The control cells had a rounded morphology and poorly defined actin stress fibers (Figure 3A-B). In contrast, the βIII-tubulin shRNA cells were flatter and displayed prominent actin stress fibers as evidenced by phalloidin staining (Figure 3C-D). To establish whether the reduced expression of βIII-tubulin was responsible for the change in cell shape, we also examined the cellular morphology of 2 individual H460 NSCLC βIII-tubulin rescue clones. Strikingly, the actin stress fibers were markedly reduced in these cells and their shape appeared to be very similar to the controls (Figure 3E-F).

**βIII-tubulin suppression inhibits tumor spheroid outgrowth, cell migration and sensitizes NSCLC cells to anoikis.**

To determine the functional significance of the altered cell morphology in βIII-tubulin knockdown NSCLC cells, we first measured their ability to grow out from tumor spheroids in 3D-culture. In this system, spheroids are formed by the self-assembly of clusters of cell colonies under anchorage-independent conditions where cell-cell interactions dominate (24). Notably, after transfer of the spheroids to a standard tissue culture plate, H460 βIII-tubulin shRNA expressing cells failed to attach and grow out from the spheroids (Figure 4A, panel II). In contrast, control shRNA cells readily attached and grew out from the spheroids (Figure 4A, panel I). The importance of high βIII-tubulin levels in regulating cell attachment and spheroid outgrowth was further highlighted in another independent NSCLC cell line (A549) that stably expresses TUBB3/βIII-tubulin shRNA. These cells displayed potent and stable knockdown of βIII-
tubulin (Supplementary Figure 7). In agreement with H460 βIII-tubulin shRNA cells, A549 βIII-tubulin knockdown cells (pRS/βIIIsh61) also showed a significant delay in attachment and spheroid outgrowth when compared to controls (Figure 4B, panels I and II). The ability of βIII-tubulin knockdown cells to adhere to an extracellular matrix (ECM) protein fibronectin (a major ECM protein expressed in the lung and tumor stroma) (25) was also measured. Detachment of tumor cells from the ECM is a critical step in the formation of metastases. We showed that suppression of βIII-tubulin in NSCLC cells induced a modest but significant increase in adhesion to fibronectin (Supplementary figure 6A). Next, we examined whether suppression of βIII-tubulin would influence cell migration. Briefly, A549 NSCLC cells stably expressing βIII-tubulin shRNA (pRS/βIIIsh61) or control shRNA (A549 pRS/Ctrlsh27) were seeded into the wells of modified boyden chambers, and cell migration was assessed 24h later (Supplementary Methods). Stable suppression of βIII-tubulin expression markedly reduced the migratory capacity of NSCLC cells (pRS/βIIIsh61 migration index of 3.95 ± 0.70) when compared to controls (A549 pRS/Ctrlsh27 migration index of 30.93 ± 4.81) (Supplementary figure 6B). Collectively, these results provide strong evidence that βIII-tubulin plays an important role in regulating cell-cell and cell-matrix interactions as well as the migration capacity of NSCLC cells.

Typically, detachment of normal epithelial cells from ECM results in programmed cell death ‘anoikis’. Tumor cells with high metastatic potential are resistant to anoikis which allows them to survive under non-adherent conditions and travel in the blood or lymph to form metastases. To determine whether suppression of βIII-tubulin modulated anoikis sensitivity, βIII-tubulin shRNA cells were plated in poly-HEMA coated culture plates
prevent cell adhesion. Cell death was then measured by annexin V staining. Suppression of βIII-tubulin did not induce cell death in adherent conditions (Figure 4C). In contrast, when kept in non-adherent conditions βIII-tubulin knockdown significantly increased anoikis sensitivity in both the pRS/βIII_{SH4} and pRS/βIII_{SH59} cell clones when compared to controls (Figure 4C). To confirm a direct correlation between βIII-tubulin expression and increased anoikis sensitivity, the same experiment was performed using the βIII-tubulin rescue (pRS/βIII_{SH4}/R17) cells. Re-expression of βIII-tubulin abolished the increased sensitivity to anoikis (Figure 4D).

**βIII-tubulin modulates AKT activity and PTEN expression in NSCLC cells**

To investigate the molecular mechanisms that mediate increased sensitivity to anoikis in βIII-tubulin knockdown cells, we examined the activation of the phosphatidylinositol 3-kinase (PI3K) downstream substrate AKT kinase. AKT is commonly dysregulated in tumor cells, and is important in promoting cell survival and resistance to anoikis (26). To examine whether there was any difference in AKT activity in βIII-tubulin shRNA expressing cells, we measured AKT phosphorylation (p-AKT) at residues S473 and T308 (phosphorylation at both sites is required for full AKT activation) between control (pRS/Ctrl_{SH2} and SH27) and βIII-tubulin knockdown (pRS/βIII_{SH4} and SH61) H460 and A549 NSCLC cells. Both βIII-tubulin shRNA expressing NSCLC cell lines displayed a clear reduction in p-AKT expression at both S473 and T308 residues when compared to controls (Figure 5A). Remarkably, restoration of βIII-tubulin back into the H460 βIII-tubulin shRNA cells completely restored p-AKT levels at both phosphorylation sites (Figure 5B). Next, the expression of the upstream tumor suppressor PTEN which is a key regulator of AKT activation was measured. Loss of PTEN is observed in many types...
of cancer including NSCLC, which often leads to increased p-AKT (27). Control cells had little PTEN expression (Figure 5C). Strikingly, NSCLC cells with stable βIII-tubulin knockdown displayed markedly increased PTEN expression (Figure 5C). Moreover, restoration of βIII-tubulin expression abolished the increase in PTEN expression, thus confirming that the low levels of βIII-tubulin were responsible for the differential expression of PTEN (Figure 5C). Finally, to determine whether the decrease in p-AKT activity in the βIII-tubulin knockdown cells, was in part mediated via increased PTEN expression, we transfected βIII-tubulin shRNA cells with PTEN siRNA (Figure 6D). Cells transfected with non-functional siRNA served as controls. Notably, the decrease in p-AKT expression levels was restored in the βIII-tubulin knockdown cells to the level of control cells (Figure 5D). These results suggest that high levels of βIII-tubulin in NSCLC cells influence p-AKT activity via PTEN.

To examine whether AKT activity is altered when NSCLC cells are exposed to non-adherent conditions, p-AKT levels were compared between the βIII-tubulin shRNA (pRS/βIII_{SH4} and SH61) and control shRNA (pRS/Ctrl{SH2} and SH27) H460 and A549 cells at differing time points when in suspension. In H460 cells, a progressive time-dependent decrease in p-AKT expression was observed in both control and βIII-tubulin shRNA cells (Figure 6A). However, p-AKT levels in βIII-tubulin shRNA (pRS/βIII_{SH4}) cells became undetectable as early as 10 minutes after exposure to non-adherent conditions, p-AKT activation in control (pRS/Ctrl_{SH2}) cells was still evident after 20 minutes in suspension (Figure 6A). In A549 cells, a marked time-dependent increase in p-AKT activation was observed in control cells (pRS/Ctrl_{SH27}), while little to no p-AKT expression was observed in the βIII-tubulin shRNA cells (pRS/βIII_{SH61}) (Figure 6A). To
determine whether the increased levels of PTEN in the βIII-tubulin knockdown cells was responsible for the suppression of Akt activation when the cells were in suspension, PTEN was silenced in βIII-tubulin knockdown cells (pRS/βIII_SH4) using siRNA. Notably, knockdown of PTEN fully restored Akt activation at all of the time points assessed (5, 10 and 20 min) in the βIII-tubulin knockdown cells (pRS/βIII_SH4) cells when compared to cells treated with control siRNA (Figure 6B). Together, these results clearly show that the activity of a pro-survival signaling protein is suppressed in NSCLC cells with low βIII-tubulin expression when exposed to non-adherent conditions, and that PTEN appears to mediate the suppression of Akt activation.

βIII-tubulin suppression increases anoikis sensitivity in vivo

To expand our findings into a clinically relevant context, and to investigate if increased sensitivity to anoikis, and reduced p-AKT activity induced by βIII-tubulin knockdown would affect the tumorigenic and/or metastatic potential of NSCLC cells, we injected control shRNA or βIII-tubulin shRNA cells into the tail vein of mice and monitored the formation of metastatic lung tumors by micro-CT imaging. After 50 days, 3/5 (60%) mice injected with control shRNA cells (pRS/Ctrl_SH2) developed lung tumors, while only one 1/5 (20%) mice injected with βIII-tubulin shRNA cells (pRS/βIII_SH4) developed lung tumors (Figure 6C). The presence of tumors within the lungs of mice was confirmed by micro-CT and histology (Figure 6D and 6E). This finding suggests that NSCLC cells with low βIII-tubulin expression have reduced tumorigenic and metastatic potential when exposed to the circulatory system.

Finally, to re-enforce that βIII-tubulin knockdown cells have a reduced capacity to form tumors in the absence of adhesion to ECM, we assessed tumor growth in mice following
subcutaneous implantation of control shRNA or βIII-tubulin shRNA cells suspended in PBS to mimic an ECM-free environment, or in matrigel to mimic attachment to ECM. After 21 days, tumors derived from control shRNA (pRS/CtrlSH2) cells which were administered in PBS were markedly bigger as compared to tumors derived from βIII-tubulin shRNA (pRS/βIII_SH4) cells in PBS (Figure 7A). In contrast, when βIII-tubulin shRNA cells were suspended in ECM we observed no significant difference in the size of the tumors generated by control (pRS/CtrlSH2) or βIII-tubulin shRNA cells (pRS/βIII_SH4) (Figure 7B). Similar results were observed when comparing another individual βIII-tubulin shRNA cell clone (pRS/βIII_SH59) and its control (pRS/CtrlSH1) (data not shown). Collectively, the data provides strong evidence in two independent mouse models of NSCLC that βIII-tubulin levels influence tumor growth via a dependence of βIII-tubulin-depleted cells on cell adhesion.
DISCUSSION

Overexpression of TUBB3/βIII-tubulin in tumor cells is often associated with resistance to chemotherapeutic drugs. Recently attention has turned to its clinical correlation with an aggressive tumor phenotype. However, despite reports highlighting βIII-tubulin as a potential biomarker for tumor aggressiveness, studies describing its functional role in tumorigenicity and metastases are limited. Herein, we report for the first time novel roles for βIII-tubulin in: 1) altering the expression of proteins involved in promoting tumor growth and metastases; 2) anoikis sensitivity; 3) modulating PTEN/AKT signaling; and 4) promoting tumor incidence and growth in vivo.

Cancer cells with high tumorigenic and metastatic potential differentially express a host of proteins including tumor suppressors, oncogenes and regulators of the cell cytoskeleton, which enable them to escape apoptosis and achieve rapid growth and motility. To gain an understanding as to whether NSCLC cells with high βIII-tubulin levels have an altered proteomic profile we performed 2D-DIGE proteomic analysis on isolated cytoplasmic and nuclear fractions from NSCLC cells with stable and potent βIII-tubulin knockdown. Our data demonstrated that βIII-tubulin can alter the expression of key proteins involved in modulating tumor growth and metastases. We demonstrated for the first time that the levels of βIII-tubulin in NSCLC cells are critical in modulating the expression of tumor suppressor proteins. For instance, maspin a member of the serpin family of serine protease inhibitors was originally identified as a tumor suppressor that is expressed in normal mammary epithelial cells, but is reduced or absent in breast tumor cells (22). Recent studies have explored the potential of maspin as a prognostic marker in different tumor types. Several reports have shown high maspin expression to be a
favorable predictor for different tumors, including NSCLC (20). However, for other tumor types such as pancreatic, gallbladder, colorectal and thyroid, high maspin expression has been associated with a poor prognosis (20). These differences are thought to be attributed to its differential expression within the cell (i.e. cytoplasmic vs. nuclear) (20). Nevertheless, in NSCLC there is an increasing body of evidence to suggest that tumors with high maspin expression have a favorable prognosis (28-30). These studies have been supported using in vitro and in vivo models of lung cancer. Beltran et al (31) demonstrated that restoration of maspin expression in NSCLC cells using artificial transcription factors combined with chromatin modifier compounds, reduced NSCLC metastatic behavior (31). Maspin was also shown to be involved in regulating the survival of lung cancer cells to chemotherapy drugs (32). Our studies showed that suppression of βIII-tubulin led to an increase in maspin expression, and that rescue of βIII-tubulin expression back into these cells was sufficient to bring maspin gene and protein expression back to control levels. This result confirms that the levels of βIII-tubulin are directly responsible for the differential expression of maspin in NSCLC cells. The regulation of maspin in tumor cells is thought to involve a number of processes including control by transcription factors such as p53, ATF-2, PTEN and Snail (33-35). Interestingly, reports have demonstrated that the carboxy-terminal tail of βIII-tubulin is subject to post-translational modifications which can allow it to form protein-protein complexes with signaling proteins such as small GTPases and/or PKC in cancer cells (36, 37). Furthermore, binding to these proteins initiated signaling cascades which promoted cellular survival under stress conditions. Therefore, it is possible that high levels of βIII-tubulin in NSCLC cells enhances protein-protein interactions which in turn
activate signaling cascades that control the expression of maspin. Further studies aimed at understanding the molecular link between βIII-tubulin and maspin in NSCLC cells are required.

Our findings showing that the levels of βIII-tubulin in NSCLC cells affects the expression of proteins involved in modulating tumorigenic and metastatic potential prompted us to explore the functional significance of βIII-tubulin in these processes. We showed that suppression of βIII-tubulin had a profound effect on the cell morphology of NSCLC cells as evidenced by their flattened appearance and prominent actin stress fibers. The altered morphology of the βIII-tubulin shRNA cells was not associated with any significant change in the expression of total actin or tubulin (results not shown).

Reorganization of the cell cytoskeleton due to loss or gain in the expression of proteins associated with the cytoskeleton has been reported in tumor cells. For instance, Bharadwaj et al (38) demonstrated that breast cancer cells with high tumorigenic and metastatic potential had reduced amounts of the actin-binding protein tropomyosin 1 (TM1) (38). Re-introduction of TM1 into these cells produced a striking alteration in the cytoskeleton with a flattened phenotype and prominent actin stress fibers. This correlated with a reduction in their tumorigenic potential by increased sensitivity to anoikis (38). Notably, our proteomic analysis identified a significant increase in TM1 expression in the βIII-tubulin shRNA cells (supplementary table 1). In addition, studies have also reported that maspin can modulate changes in the expression of proteins associated with the cytoskeleton which correlate to increased cell adhesion and a reduced migratory phenotype (39). We cannot exclude the possibility that maspin may be acting in concert with other proteins and it is possible that both TM1 and maspin may
in part be involved in the re-organization of the cell cytoskeleton in NSCLC cells with suppressed βIII-tubulin.

Cancer cells must detach from an ECM matrix, survive under anchorage-independent conditions to travel in the blood or lymphatic system, and adhere and proliferate at new organ sites (40). Therefore, tumor cells with metastatic potential have acquired altered mechanisms of cellular adhesion as well as resistance to anoikis (40, 41). We demonstrated that suppression of βIII-tubulin prevented / delayed the ability of NSCLC cells to adhere and grow out from multicellular tumor spheroids. In addition, we showed that βIII-tubulin knockdown increased cell adhesion to fibronectin. These results support the notion that βIII-tubulin plays an important role in regulating cell-cell and cell-matrix interactions. We also demonstrated that suppression of βIII-tubulin sensitized NSCLC cells to anoikis. Importantly, anoikis was reversed by re-expression of βIII-tubulin indicating a direct correlation between βIII-tubulin levels and anoikis sensitivity. To gain an understanding into the mechanisms that link βIII-tubulin knockdown and increased anoikis, we examined the activation of the pro-survival signaling protein AKT in βIII-tubulin knockdown cells. There are numerous reports which highlight the importance of the AKT signaling pathway in tumor cells as a mechanism that promotes resistance to various forms of apoptosis (40). Interestingly, we showed that suppression of βIII-tubulin correlated with a decrease in AKT phosphorylation at both phosphorylation sites (S473 and T308). Strikingly, AKT phosphorylation was fully restored following re-expression of βIII-tubulin. One of the key regulators of AKT activity is the tumor suppressor PTEN. The principal catalytic function of this phosphatase is to dephosphorylate phosphatidylinositol-triphosphate which is a potent activator of AKT (42). Loss of PTEN
function leads to AKT signaling hyperactivation and is a common feature in a wide range of tumors (43). In agreement with our finding that inhibition of βIII-tubulin expression correlates with decreased AKT phosphorylation, we showed that expression of PTEN is markedly increased in βIII-tubulin shRNA expressing cells. Furthermore, knockdown of PTEN using siRNA in the βIII-tubulin shRNA cells restored p-AKT activity back to controls. Finally, we showed that when kept in non-adherent conditions which is a surrogate marker for tumorigenicity and is an environment in which cells are susceptible to anoikis, AKT activation in βIII-tubulin shRNA cells became undetectable much earlier compared to control cells. The question that arises is how increased levels of βIII-tubulin influence the activity of an important pro-survival signaling pathway in NSCLC cells. Although more studies will be needed to fully answer this question, it should be noted, that silencing PTEN in the βIII-tubulin shRNA cells restored AKT phosphorylation when the cells were exposed to adherent-or non-adherent conditions. A recent study by Nam et al (32) showed that over-expression of maspin in NSCLC cells resulted in a significant reduction in AKT phosphorylation which correlated with increased expression of PTEN (32). Therefore, it is tempting to speculate that the expression levels of βIII-tubulin, maspin and PTEN are important determinants in modulating AKT activity in NSCLC cells. This in turn may not only be influencing tumor cell survival under stressful conditions such as nutrient and oxygen deprivation, but also under chemotherapeutetic insult. Given the complex multifunctional nature of βIII-tubulin it is highly likely there are other additional biological effects on NSCLC cells.

Finally, the importance of βIII-tubulin in promoting tumorigenicity and metastases in NSCLC cells was highlighted in two different lung cancer mouse models. The first
model measured the ability of βIII-tubulin shRNA cells to form metastatic lung tumors following dissemination through the vascular system. In this model, tumor cells need to be able to survive in an anchorage-independent environment before extravasating and seeding in organs. In this case expression of βIII-tubulin correlated with increased metastatic potential as suppression of this protein led to decreased tumor dissemination. The second model assessed tumor growth when the cells were administered in the presence or absence of ECM. Here it became clearly evident that differences in the tumorigenic potential between βIII-tubulin shRNA and control shRNA cells could only be observed if cells are exposed to a non-adherent environment.

Collectively, this work provides valuable biological insight into the multifunctional role of βIII-tubulin in regulating tumor suppressor and metastasis pathways as well as pro-survival signaling activity to directly influence NSCLC tumor growth and incidence.
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FIGURE LEGENDS

Figure 1: Suppression of βIII-tubulin increases the expression of maspin in NSCLC cells. (A) Representative 2D-DIGE densitometric volume of maspin is shown in a 3D-view generated by the DeCyder Image Analysis software package. Arrows indicate the 3D-densitometric peak for maspin expression in both control (H460 pRS/CtrlSH1 and SH2) and βIII-tubulin (H460 pRS/βIII SH4 and SH59) shRNA expressing NSCLC cells. (B) Western blot and densitometry graph demonstrating that potent suppression of βIII-tubulin expression in 2 individual βIII-tubulin shRNA NSCLC cell clones (H460 pRS/βIII SH4 and SH59) is associated with a significant increase in maspin protein expression when compared to 2 individual control clones (H460 pRS/CtrlSH1 and SH2), n = 3-5 independent experiments all samples were normalized to the housekeeping protein GAPDH (*p<0.001, H460 pRS/βIII SH4 and SH59 vs. H460 pRS/CtrlSH1 and SH2). (C) Western blot showing an increase in maspin protein expression in both the cytoplasmic and nuclear fractions of NSCLC cells stably expressing βIII-tubulin shRNA (H460 pRS/βIII SH4 and SH59) when compared to 2 individual control clones (H460 pRS/CtrlSH1 and SH2), n=3 independent experiments. (D) A graph showing a significant increase in maspin mRNA expression in 2 individual cell clones of NSCLC cells stably expressing βIII-tubulin shRNA (H460 pRS/βIII SH4 and SH59) when compared to control clones (pRS/CtrlSH1 and SH2), n = 4 separate experiments, all samples were normalized to the housekeeping gene β2-microglobulin (*p<0.001, H460 pRS/βIII SH4 and SH59 vs. pRS/CtrlSH1 and SH2).

Figure 2: Rescue of βIII-tubulin reverses the increase in maspin expression in βIII-tubulin shRNA NSCLC expressing cells. (A) Representative western blot and
densitometry graph showing that in 2 individual βIII-tubulin rescue cell clones (H460 pRS/βIII R6 and pRS/βIII R17), maspin protein expression is returned back to control (pRS/CtrlSH1 and SH2) levels when compared to βIII-tubulin shRNA (H460 pRS/βIII SH4) or empty vector control (H460 pRS/EV), n = 6 independent experiments, all samples were normalized to the housekeeping protein GAPDH. (*p<0.001, H460 pRS/βIII SH4 and pRS/βIII EV vs. control clones H460 pRS/CtrlSH1 and SH2; #p<0.001, H460 pRS/βIII R6 and R17 vs. pRS/βIII SH4 and pRS/βIII EV). B) A graph showing that maspin mRNA expression levels are returned to control (H460 pRS/CtrlSH1 and SH2) and empty vector control (H460 pRS/EV) in 2 individual βIII-tubulin rescue cell clones (H460 pRS/βIII R6 and pRS/βIII R17), n = 4 independent experiments, all samples were normalized to the housekeeping gene β2-microglobulin (*p<0.001, H460 pRS/βIII SH4 and pRS/βIII EV vs. control clones pRS/CtrlSH1 and SH2; #p<0.001, pRS/βIII R6 and R17 vs. pRS/βIII SH4 and pRS/βIII EV).

Figure 3: Suppression of βIII-tubulin alters the cell morphology of NSCLC cells. Representative microscopy images of phalloidin (F-actin) staining on βIII-tubulin shRNA expressing NSCLC (H460 pRS/βIII SH4 and SH59) cells (panels C and D) demonstrating a marked alteration in cell morphology as evidenced by a flattened appearance with prominent actin stress fibers (arrows indicate actin stress fibres) when compared to control shRNA expressing NSCLC cells (H460 pRS/CtrlSH1 and SH2) (panels A and B). Rescue of βIII-tubulin expression (panels E and F) (H460 pRS/βIII R6 and R17) reduced the presence of actin stress fibers. Scale bar = 20μm, n = 3 independent experiments.

Figure 4: Suppression of βIII-tubulin decreases tumor spheroid outgrowth and induces anoikis in NSCLC cells. A) and B) Representative photomicrograph images and graphs showing a significant delay in the attachment and outgrowth of H460 and
A549 NSCLC cells stably expressing βIII-tubulin shRNA (H460 pRS/βIII_{SH4} and A549 pRS/βIII_{SH61}) compared to controls (H460 pRS/Ctrl_{SH2} and A549 pRS/Ctrl_{SH27}) 24h post-attachment, n = 3 experiments, *p<0.05. ** Graph demonstrating a significant increase in anoikis (cell-detachment induced cell death) in NSCLC cells stably expressing βIII-tubulin shRNA (H460 pRS/βIII_{SH4} and SH59) when compared to controls (H460 pRS/Ctrl_{SH1} and SH2) 48h post-seeding, n=3 independent experiments (*p<0.001, adherent vs. suspension; # p<0.001 pRS/βIII_{SH4} and SH59 vs. pRS/Ctrl_{SH1} and SH2 in suspension). 

** Figure 5: Suppression of βIII-tubulin modulates AKT activity and PTEN expression in NSCLC cells.** 

**A)** Representative western blots demonstrating that βIII-tubulin shRNA expressing cells (H460 pRS/βIII_{SH4} and A549 pRS/βIII_{SH61}) have significantly decreased phosphorylation of AKT at S473 and T308 residues vs. control (H460 pRS/Ctrl_{SH2} and A549 pRS/Ctrl_{SH27}) cells. Total Akt levels were unchanged. 

**B)** Representative western blot showing that re-expression of βIII-tubulin (H460 pRS/βIII R17) back into βIII-tubulin shRNA (H460 pRS/βIII_{SH4}) cells restores p-AKT activity at both S473 and T308 residues back to control (H460 pRS/Ctrl_{SH2}). 

**C)** Representative western blot demonstrating increased PTEN expression in βIII-tubulin shRNA (H460 pRS/βIII_{SH4}) cells when compared to control (H460 pRS/Ctrl_{SH2}) cells. Restoration of βIII-tubulin expression (H460 pRS/βIII R17) returns PTEN expression back to control (H460 pRS/Ctrl_{SH2}). Total AKT levels were unchanged. 

**D)** Representative western blot
and densitometry graph showing restoration of p-AKT S473 expression in βIII-tubulin shRNA (H460 pRS/βIII_SH4) cells, 72h post-transfection with PTEN siRNA. Cells transfected with non-functional siRNA served as controls. Total AKT levels were unchanged. *p<0.05. GAPDH was used a protein loading control for all western blots. All data is representative of n=3 independent experiments.

**Figure 6:** Suppression of βIII-tubulin reduces tumor incidence in vivo. 

A) Representative western blots from βIII-tubulin shRNA (H460 pRS/βIII_SH4 and A549 pRS/βIII_SH61) NSCLC cells showing a decrease in p-AKT (S473) expression levels at different time (5, 10, 20 min) points when exposed to non-adherent conditions. Total AKT levels were unchanged. GAPDH was used as a protein loading control, n=3 independent experiments. 

B) Representative western blot showing that knockdown of PTEN in βIII-tubulin shRNA H460 (pRS/βIII_SH4) cells using siRNA, completely reverses the decrease in p-AKT (S473) expression over time (5, 10, 20 min) when exposed to non-adherent condition. Cells (pRS/βIII_SH4) treated with non-functional siRNA served as controls, n = 3 independent experiments. 

C) Graphic representation of tumor incidence for control (H460 pRS/Ctrl_SH2) and βIII-tubulin shRNA (H460 pRS/βIII_SH4) cells, n = 5 animals per group. 

D) Representative micro-CT images of lung tumors (arrowheads) on the axial, coronal and sagittal planes 50 days after tail vein injection of control (H460 pRS/Ctrl_SH2) or βIII-tubulin shRNA (H460 pRS/βIII_SH4) NSCLC cells. 

E) Representative hematoxylin and eosin stain showing the presence of tumors within the lung of mice injected systemically with control shRNA (H460 pRS/Ctrl_SH2) or βIII-tubulin shRNA (H460 pRS/βIII_SH4) NSCLC cells. T = tumor, NL = normal lung and H = heart.
Figure 7: Suppression of βIII-tubulin reduces tumor growth in an anchorage-independent environment. A) Graph showing tumour volumes (mm$^3$) following subcutaneous injection of control shRNA (pRS/Ctrl SH2) or βIII-tubulin shRNA NSCLC cells (pRS/βIII$_{SH4}$) when administered in an anchorage-independent environment (PBS).

B) Graph demonstrating tumor volumes (mm$^3$) following subcutaneous injection of control shRNA (pRS/Ctrl SH2) or βIII-tubulin shRNA NSCLC cells (pRS/βIII$_{SH4}$) when administered in an anchorage-dependent environment (matrigel), n= 3-5 mice for βIII-tubulin knockdown and control cells in PBS; n= 6-7 animals for βIII-tubulin knockdown and control cells in matrigel; values presented as mean ± SEM.
Figure 1

A) 

B) 

C) 

D)
Figure 2

A) Western blot analysis of βIII-tubulin, Maspin, and GAPDH in different cell lines.

B) Bar graph showing Maspin mRNA levels (Relative to Ctrl_SH1) in different cell lines.

* indicates significant difference compared to Ctrl_SH1 and 2.
# indicates significant difference compared to pRS/βIII_SH4.
Figure 3

A) pRS/Ctrl3SH1

B) pRS/Ctrl3SH2

C) pRS/βIII3SH4

D) pRS/βIII3SH59

E) pRS/βIII R6

F) pRS/βIII R17
Figure 4

A) 24h post-attachment  H460 NSCLC cells

B) 24h post-attachment  A549 NSCLC cells

C) % Cell Death

D) % Cell Death

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### Figure 6

#### A) H460 cells

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#### B) pRS/βIII<sub>SH4</sub>

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#### C) Number of mice with lung tumors

- pRS/Ctrl<sub>SH2</sub>: 60%
- pRS/βIII<sub>SH4</sub>: 20%

#### D) Imaging

- Axial
- Coronal
- Sagittal

#### E) Histology

- pRS/Ctrl<sub>SH2</sub>
- pRS/βIII<sub>SH4</sub>
Figure 7

A) Anchorage-Independent (PBS)

- pRS/Ctrl$_{SH2}$, PBS
- pRS/$\beta$III$_{SH4}$, PBS

B) Anchorage-Dependent (Matrigel)

- pRS/Ctrl$_{SH2}$, Matrigel
- pRS/$\beta$III$_{SH4}$, Matrigel

Tumor Volume (mm$^3$) vs Days post injection
TUBB3/βIII-tubulin acts through the PTEN/AKT signaling axis to promote tumorigenesis and anoikis resistance in non-small cell lung cancer.

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