in this study, this correlation could not be established between PAX3 loss and apoptotic induction. PAX3-dependent apoptosis has been reported in melanoma cells, however, and is indicated with a dotted arrow (20, 24).

**Supplementary Information**

**Supplementary Materials and Methods**

**Melanoma Lysate Kinase Assay**

To ensure that pGex2T-PAX3PDHD-WT could be phosphorylated by GSK-3β within cell lysates, several melanoma cell lines (A375, SKMEL-23, SKMEL-28, 537, 624, and 888) were cultured for 72 hours without treatment, with carrier alone or with 20μM SB216763. An equal number of cells were lysed in CelLytic express buffer (Sigma) supplemented with 10μl/ml each of protease inhibitor cocktail, phosphatase inhibitor cocktail I and II (Sigma). Total lysate protein concentrations were quantified with the DC Protein Assay (BioRad). Equal amounts of total lysate protein (100μg) were added to GST-tagged pGex2T-PAX3PDHD-WT-containing beads in ATP cocktail mix (25mM MOPS, pH7.2, 12.5mM β-glycerol phosphate, 25mM $MgCl_2$, 5mM EGTA, 2mM EDTA, 0.25mM DTT, and 0.25mM ATP), and ATP [$\gamma$-$P^{32}$]. The mixture was incubated at 30°C on a rotator for 30 minutes, washed thrice and resuspended in loading buffer prior to running on a 4-12% Bis-Tris gel (Invitrogen). The gel was dried and autoradiography was performed. Equal gel loading was verified by running an aliquot of each sample on a 4-12% Bis-Tris gel then transferring the protein onto nitrocellulose membrane (BioRad). The membrane was probed with PAX3 antibody (1:1000, Calbiochem).

**Tandem mass spectrometry (MS/MS)**

The band to be analyzed was excised and divided into 1 $mm^3$ pieces and completely destained using 100mM ammonium bicarbonate pH 7.5 in 50% acetonitrile. A reduction step was performed with 50mM ammonium bicarbonate pH 7.5 and 200mM TCEP (Tris (2-carboxyethyl)phosphine HCl) at 37 ºC for 30 minutes. The sample was alkylated with 50mM iodoacetamide and allowed to react in the dark at 20°C for 30 minutes. Gel pieces were washed in water, dehydrated with acetonitrile then dried by SpeedVac for 30 minutes. Trypsin digestion was carried out overnight at 37ºC using (1:50) enzyme to protein ratio of sequencing grade-modified trypsin (Promega), in 50mM ammonium bicarbonate pH7.5, and 20mM $CaCl_2$. Peptides were extracted from the gel pieces with 5% formic acid and dried in a SpeedVac.

For orbitrap $MS^2/MS^3$, the peptide samples were loaded to a C8 trapping cartridge OptiPak custom-packed with Michrom Magic C8, 5u, 200A (Optimize Technologies), washed, then switched in-line with a 20cm by 75um C18 'packed spray tip' nano column packed with Michrom Magic C18AQ, 5u, 200A (Michrom BioResources), for a 2-step gradient. Using a flowrate of 350nl/min, a 90 min, 2-step LC gradient was run from 5%B to 50%B in 60 min, followed by 50%-95%B over the next 10 minutes, hold 10 minutes at 95% B, back to starting conditions and re-equilibrated where mobile phase A is water/acetonitrile/formic acid 98/2/0.2 and mobile phase B is acetonitrile/isopropanol/water/formic acid 80/10/10/0.2.

The samples were analyzed via electrospray tandem mass spectrometry (LC-MS/MS) on a linear ion trap in tandem with an Orbitrap (LTQ-Orbitrap) mass spectrometer

(Thermo Scientific). The peptide sample was injected into the Orbitrap using a spray voltage of 2.25kV in positive ion mode for data-dependent $MS^2/MS^3$ analysis. Full mass scans were acquired using an Orbitrap (375-1950m/z, mass resolution = 60,000), with lockmasses, followed by $MS^2$ scans in the LTQ of the 7 most intense doubly and triply charged-only precursor ions followed by neutral loss of $H_3PO_4$ triggered $MS^3$ scans in the LTQ. The normalized collision energy for CID was set at 31 for $MS^2$ scans and 35 for $MS^3$ scans. Ions selected for $MS^2$ analysis were subsequently placed on an exclusion list for 60 seconds using an isolation width of 1.6Da, and low mass exclusion of 0.8Da, high mass exclusion of 0.8Da.

**Database searching**

All MS/MS samples were analyzed using Mascot (Matrix Science; version 2.3) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). X!Tandem was set up to search Subset_IPI_mouse_3.84_3972898712270266549.fasta (Version 3.84, 60107 entries) Subset_ cRAP_20100324.fasta _952128198969618358.fasta (Version 20100324, 782 entries). Mascot was set up to search cRAP_20100324.fasta (Version 20100324, 782 entries) and IPI_mouse_3.84.fasta (Version 3.84, 60107 entries) assuming the digestion enzyme trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 20 PPM and with a relaxed parent ion tolerance of 4Da. Iodoacetamide derivative of cysteine was specified in as a fixed modification. Dehydro of serine and threonine, oxidation of methionine, n-Formylation of

the n-terminus and phosphorylation of serine and threonine were specified as variable modifications.

**Criteria for protein identification**

Scaffold (version Scaffold_3_00_08, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (1). Peptide probability was relaxed to 0% to look for any potential phosphorylation modifications. All phosphorylation-modified peptides were manually validated by the criteria mentioned previously. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (2). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

The following criteria were used to manually evaluate and identify sites of phosphorylation: 1) MS tolerance was held to 20ppm and unidentified spectra were re-searched with a 4Da MS window. 2) Ser(P) and Thr(P) were required to show neutral loss of the $H_3PO_4$ from the precursor and/or fragment ions or trigger a $MS^3$ scan. 3) Peptides were required to be >6 amino acids in length. 4) Extensive coverage of b/y ion series from the CID fragmentation. 5) All proline-containing peptides were required to show cleavage N-terminally to the proline residue. Manual analysis of the data by

multiple platforms (Mascot, Scaffold, X!Tandem) as well as evidence of dihydro-alanine (dHA) residue reduction from MS2 to MS3 measurements were utilized to provide support for the identification of phosphorylated residues.

**Supplementary Figure S1.** Verification of *PAX3*, *GSK-3α*, and *GSK-3β* siRNA specificity. A, sequence of the siRNA oligos against *GSK-3α* (α1-α4) and *GSK-3β* (β1-β4). B, western blot of siRNA oligos. 537 cells were transfected with the siScramble control, siRNA oligos α1-α4 and siRNA oligos β1-β4. The siRNA oligos against each isoform reduce that specific protein without altering the levels of the other isoform. Based on these results, we chose oligos α2 and β4 for further experimentation. C, sequence of the siRNA oligos against *PAX3* (si*PAX3* A-D). D, Western blot to test si*PAX3* oligos. All 4 *PAX3* siRNAs reduced PAX3 levels but oligo A had the greatest effect and was therefore selected for future experimentation.

**Supplementary Figure S2.** Treatment of SKMEL-23 and 537 cells with SB216763 leads to an increase in cells in the $G_2$ phase of the cell cycle. A-H, cell cycle analysis by flow cytometry. SKMEL-23 (A-D) and 537 (E-H) cells were left untreated, treated with DMSO, with 10μM SB216763 or 20μM SB216763 for 30 hours and analyzed by flow-cytometry for cell cycle progression. DNA from cells in $G_0/G_1$ was measured as 2N and $G_2$ DNA was measured as 4N. SKMEL-23 cells left untreated (A) and treated with carrier alone (B) exhibited similar proportions of the total population in $G_2$ phase. SKMEL-23 cells treated with 10μM SB216763 (C) and 20μM SB216763 (D)

demonstrated a dose-dependent increase in the proportion of cells in $G_2$. Similarly, 537 cells left untreated (E) and treated with DMSO (F) possessed a low percentage of the total population in $G_2$ compared to those treated with 10μM SB216763 (G) and 20μM SB216763 (H), which had an overall increase in the percentage of cells in the $G_2$ phase. Graphical representations of percentage of cells in the $G_2$ peak for each experimental group of SKMEL-23 (I) and 537 (J) cells. Values are means +/- s.d. (n=3).

**Supplementary Figure S3.** GSK-3 within total melanoma cell lysates phosphorylates PAX3. A, A375, 888, SKMEL-23, 537, 624, and SKMEL-28 cells were untreated, treated with DMSO or with 20μM SB216763 for 72 hours. Lysate normalized by total cell number was used in the kinase assay mixed with PAX3PDHD-WT. The kinase assays were run on a gel, which was dried and subjected to autoradiography (top row for each cell type). Auto-phosphorylation band is seen on the top (*) and the band representing PAX3 is on the bottom. PAX3 is phosphorylated in the untreated and DMSO-treated cells but is less abundant in the GSK-3 inhibitor-treated cells. A fraction of each kinase assay was probed for PAX3 as a loading control by Western analysis (bottom row for each cell type). B, densitometry readings on phosphorylated PAX3 graphed as percent band intensity as compared to controls. Values are means +/- s.d. (n=3 independent experiments).

**Supplementary Figure S4.** Mascot search and analysis of phospho-PAX3 peptides.

**Supplementary Figure S5.** Verification of PAX3-HA expression in SKMEL-23 and 537 cells. SKMEL-23 and 537 cells were transfected with either pcDNA3 or pcDNA3-PAX3-HA and selected for transfected cells with Geneticin. After selection, cells were left untreated or treated with carrier or SB216763 for 72 hours. Blots were probed with HA or vinculin antibody as a loading control.

## Supplementary References

1. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 2002;74:5383-92.
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