APPENDICES

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Appendix 1. Vector Maps for DNA constructs used in this thesis.

A) pBABE GFP constructs contained SV40 sequences.

B) pBABE puro constructs contained H-Ras-V12 and PP2A-α muts.

C) pMKO GFP constructs contained shRNA sequences against PP2A-α and regulatory B subunits.

All constructs and these vector maps were originally obtained from Addgene inc (www.addgene.org)
Appendix 2a Correlation of protein expression and mRNA expression for PP2A subunits.

Average protein expression was plotted against average mRNA expression for all cell lines, and analysed by linear regression.
Appendix 2b Correlation of protein expression and mRNA expression for PP2A subunits.

Average protein expression was plotted against average mRNA expression for all cell lines, and analysed by linear regression.
Appendix 2c Correlation of protein expression and mRNA expression for PP2A subunits.
Average protein expression was plotted against average mRNA expression for all cell lines, and analysed by linear regression.
Appendix 2d Correlation of protein expression and mRNA expression for PP2A subunits.

Average protein expression was plotted against average mRNA expression for all cell lines, and analysed by linear regression.
Appendix 3a Ki67 staining shows proliferation in sub-confluent breast and breast cancer cells, but not in confluent cells.

Cells were grown on glass coverslips; fixed, permeabilised and dividing cells detected with anti-Ki67 antibody, followed by secondary fluorescently labelled antibody. Nuclei were labelled with DAPI (blue). Scale bar = 50μm. Sub-confluent cells are on the left, and confluent cultures on the right for each cell line.
Appendix 3b Ki67 staining shows proliferation in sub-confluent breast and breast cancer cells, but not in confluent cells. Cells were grown on glass coverslips; fixed, permeabilised and dividing cells detected with anti-Ki67 antibody, followed by secondary fluorescently labelled antibody. Nuclei were imaged with DAPI (blue). Scale bar = 50μm. Sub-confluent cells are on the left, and confluent cultures on the right for each cell line.
Appendix 4 PP2A-A co-localises with β4 integrin in primary human mammary epithelial cells.

HMEC cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and β4 integrin (green) co-localise (yellow on merge image) at the periphery of HMEC cells.
Appendix 5a PP2A is not expressed in the nuclei in confluent breast and breast cancer cell lines. Cells were grown on glass coverslips until they reached confluence; then were fixed, permeabilised and proteins detected with specific anti-PP2A antibodies as indicated, followed by secondary fluorescent antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.
Appendix 5b PP2A is not expressed in the nuclei in confluent breast and breast cancer cell lines. Cells were grown on glass coverslips until they reached confluence; then were fixed, permeabilised and proteins detected with specific anti-PP2A antibodies as indicated, followed by secondary fluorescent antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50 μm. Note for HMT-3522-T42 cells, the exposure time had to be reduced in order to see any cellular structures. All other images are taken at the same exposure.
Appendix 6 PP2A-A co-localises with β4 integrin at the periphery of HMT-3522-T42 cells.

Cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and β4 integrin (green) co-localise (yellow on merge image) at the periphery of HMT-3522-T42 cells.
Appendix 7  MDA-MB-231 cells did not spread on glass coverslips as on tissue culture plastic. As MDA-MB-231 cells did not spread on glass coverslips and have the same morphology as when cultured on tissue culture plastic, PP2A expression by immunofluorescence (Figure 3.20) must be interpreted with caution.
Appendix 8a PP2A-A does not co-localise with proliferating cells in the outer cell layer of MCF10A acini – Day 8.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) does not co-localise with a marker of cellular proliferation, Ki67 (green) in day 8 acini.
Appendix 8b PP2A-C does not co-localise with proliferating cells in the outer cell layer of MCF10A acini – Day 14.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-C (red) does not co-localise with a marker of cellular proliferation, Ki67 (green) in day 14 acini.
Appendix 9 PP2A-C protein expression in a breast cancer tissue array.
Human tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using an anti-PP2A-C antibody (brown). Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm.
Appendix 10 Negative control slides incubated with isotype matched antibodies for human tissue array analysis by immunohistochemistry.

Negative controls for anti-PP2A antibodies were isotype matched immunoglobulins. Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope.

A) Negative control slide for anti-PP2A-A antibody.
B) Negative control slide for anti-PP2A-C antibody.
Appendix 11a PP2A-A is not over-expressed in the nuclei of breast tissue.
Human tissue arrays were analysed by immunohistochemistry with an anti-PP2A-A antibody (brown). Nuclei are stained with hematoxylin (blue).
Appendix 11b PP2A-A is not over-expressed in the nuclei of breast tissue.
Human tissue arrays were analysed by immunohistochemistry with an anti-PP2A-A antibody (brown). Nuclei are stained with hematoxylin (blue).
Appendix 11c PP2A-C is not over-expressed in the nuclei of breast tissue.
Human tissue arrays were analysed by immunohistochemistry with an anti-PP2A-AC antibody (brown). Nuclei are stained with hematoxylin (blue).
Appendix 11d PP2A-C is expressed in the nucleus of myoepithelial cells and also the tumour tissue from this patient.

Human tissue arrays were analysed by immunohistochemistry with an anti-PP2A-C antibody (brown). Nuclei are stained with hematoxylin (blue).
Appendix 12 Patient data for tissue arrays with PP2A subunit IHC scores.

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Spot numbers (#) correspond to those shown in Figure 3.38. TNM and Stage: AJCC Cancer staging manual (6th Ed). LN = lymph nodes positive lymph nodes/lymph nodes examined. ER = Estrogen receptor, PR = Progesterone receptor.
Appendix 13 Regions of analysis for PP2A-A expression in breast tumour arrays.

Human tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A antibodies. Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. For normal epithelium cores and also tumour samples that were not homogenous, ducts or tumour tissues were traced prior to analysis. Note that for some cores with areas of tissue damage, negative tracing was used to exclude these regions – these are dashed lines on tracings.
Appendix 14 PP2A-B'γ is highly expressed in plasma cells with considerable levels of background staining.

A) Breast tumour core stained with PP2A-B'γ (brown) and nuclei are stained with hematoxylin (blue). Arrows indicate plasma cells as identified by a pathologist. In comparison the levels of tumour cell staining would be considered background staining.

B) PP2A-B'γ (brown) only as for (A).

C) An example of PP2A-B'γ staining in a breast tumour core (#33) with tumour tissue (left) which demonstrates lower intensity staining compared with normal ducts (right). Below each enlargement is a colour representation of the staining intensity, with dark blue = negative, yellow = 1+, orange = 2+ and red = 3+. 
Appendix 15 Apoptotic appearance of MCF10A cells with PP2A-Aα expression suppressed below 20%.

MCF10A cells were transduced with an shRNA sequence that knocks down PP2A-Aα expression. This clone had less than 20% expression compared to untransduced MCF10A cells, proliferated very slowly and eventually died. Arrows indicate cells that seem to have apoptotic vacuoles.
Appendix 16 MCF10A cells infected with scrambled shRNA constructs have an altered cellular morphology compared to untransduced MCF10A cells.

MCF10A cells were transduced with an shRNA sequence that does not encode any known protein sequence. However, for an unknown reason these cells had an altered cellular phenotype under normal cell culture conditions.
Appendix 17 PP2A-Bα shRNA acini have small, but cleared lobes protruding from the central acini.

MCF10A-Bα shRNA cells were cultured from single cells on extracellular matrix proteins for 20 days as a model of mammary gland development. Acini were fixed, permeabilised and nuclei were labelled with DAPI (blue). Scale bar = 50μm. Lobes are marked with white arrows.
Appendix 18a Proliferation rate of PP2A-Aα mutant acini demonstrating a lobular phenotype.

Acini were cultured from single cells on a bed of extracellular matrix proteins for 20 days as a model of mammary gland development.

A) Acini were fixed, permeabilised and proteins detected with anti-Ki67 antibody as a marker of cellular proliferation, followed by secondary fluorescently labelled antibody (red). Nuclei were labelled with DAPI (blue). Scale bar = 50 μm. Green arrows indicate lobular phenotype, purple arrows indicate elongated phenotype. Lobular phenotype have more proliferating cells compared to elongated phenotype.

B-D) The proliferation rate of 3D cultures was determined by counting the number of Ki67 positive cells per acini, and plotting according to acini morphology. Error bars are standard error of the mean. *p<0.05, **p<0.01, ***p<0.001 compared to normal morphology for each cell line using a students t-test, or as indicated by horizontal bars in E64D #5. Lobular phenotype have more proliferating cells compared to elongated phenotype for all PP2A-Aα mutants with a predominantly lobular phenotype: E64D #5 (B), E64G #4 (C) and P179A #4 (D).
Appendix 18b Proliferation rate of PP2A-Aα mutant acini demonstrating an elongated phenotype.

Acini were cultured from single cells on a bed of extracellular matrix proteins for 20 days as a model of mammary gland development. The proliferation rate of 3D cultures was determined by counting the number of Ki67 positive cells per acini, and plotting according to acini morphology. Error bars are standard error of the mean. **p<0.01, ***p<0.001 compared to normal morphology for each cell line using a students t-test.

The elongated phenotype have a higher proliferation rate than normal acini, but are less proliferative than the lobular phenotype in Appendix 17a.
Appendix 18c Proliferation rate of PP2A-Aα R418W mutant acini.

Acini were cultured from single cells on a bed of extracellular matrix proteins for 20 days as a model of mammary gland development.

A) The proliferation rate of 3D cultures was determined by counting the number of Ki67 positive cells per acini, and plotting according to acini morphology. Error bars are standard error of the mean. *p<0.05, ***p<0.001 compared to normal morphology for each cell line using a students t-test. The few invasive acini have more proliferating cells compared to both the lobular and elongated phenotypes.

B) Acini were fixed, permeabilised and proteins detected with anti-Ki67 antibody as a marker of cellular proliferation, followed by secondary fluorescently labelled antibody (red). Nuclei were labelled with DAPI (blue). Scale bar = 50μm. Green arrows indicate lobular, purple arrows indicate elongated and orange arrows indicate invasive phenotypes.
Appendix 19 PP2A co-localises with integrins at the migrating edge of an MCF10A wound healing assay.

Confluent monolayers of MCF10A cells were scratched with pipette tip and then allowed to begin migrating overnight. The following day, cells were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and β4 integrin (green) are upregulated at the migrating edge of a wound.
Appendix 20 PP2A-α R418W mutants produced a few structures that were very invasive in appearance.

Acini were cultured from single cells on a bed of extracellular matrix proteins for up to 20 days as a model of mammary gland development. In both of the R418W clones examined, a few structures with an invasive appearance, which were able to grow full acini-type structures from the ends of the larger structure were observed. As these structures were very large, they were destroyed during the numerous washing procedures associated with immunofluorescence staining and thus were unable to examined for PP2A expression.
REFERENCES


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Mukhopadhyay, A., S. A. Saddoughi, et al. (2009). "Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the


