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The Effects of Atypical Protein Kinase C on TGFβ Signalling

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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THE EFFECTS OF ATYPICAL PROTEIN KINASE C ON TGFβ SIGNALLING

(Thesis format: Integrated Article)

by

Adrian Gunaratne

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The transforming growth factor beta (TGFβ) signalling pathway is an essential regulator of many cellular processes including epithelial growth control, epithelial to mesenchymal transition (EMT), apoptosis, and the establishment of developmental fate. Alterations in TGFβ signalling patterns are associated with various pathological disorders such as fibrosis and cancer. In recent years it has become clear that regulation of TGFβ signalling is dependent on the trafficking and endocytosis of the TGFβ receptors, however, the factors that control these processes are still under investigation.

In this thesis, I examined the role of Protein Kinase C (PKC) in the regulation of TGFβ signalling pathways and found that the Atypical PKC isoforms (aPKC; a subgroup of the PKC family) indeed can alter TGFβ receptor signalling. My work has shown that the modulation of aPKC expression or activity using inhibitors and/or small interfering RNA (siRNA) prolongs the temporal phosphorylation of the downstream transcription factor Smad2 through altered receptor membrane trafficking. Furthermore, I showed that aPKC activity and expression alters the phosphorylation and degradation of Par6, which in turn affects TGFβ induced EMT and migration. Finally, I examined global gene expression changes in aPKC silenced cells - and related these effects to altered Smad nuclear accumulation. Notably, we also found that these cells demonstrate enhanced p38 MAPK signalling, which sensitizes them to TGFβ induced apoptotic response.

In conclusion, I found that aPKC isoform activity and expression is intricately linked to the regulation of various TGFβ receptor signalling pathways that control gene expression, EMT, and apoptosis.
Keywords

Transforming growth factor beta (TGFβ)

Protein Kinase C (PKC)

Atypical Protein Kinase C (aPKC)

Smad signalling

Non-Smad signalling

Par6

Epithelial to mesenchymal transition (EMT)

p38 MAPK mitogen activated protein kinase

Intracellular trafficking

Endocytosis
Co-Authorship Statement


This peer reviewed paper is entitled “Regulation of TGFβ receptor trafficking and signalling by atypical protein kinase C”. The sources of reagents and plasmids are listed in the materials and methods sections of the chapter and any additional items mentioned in the footnotes section. Figure 1 was composed by Dr. Hassina Benchabane from work she had completed in Dr. Jeff Wrana’s laboratory (University of Toronto). Figure 2 was composed by Dr. Gianni M. Di Guglielmo. These experiments were repeated by Adrian Gunaratne, and all other experiments and figures in the chapter were completed by Adrian Gunaratne.

Chapter 3 of this thesis was published in Molecular and Cellular Biology in Mar. 2013 (Mol Cell Biol. 2013 Mar;33(5):874-86.)

This peer reviewed paper is entitled “Atypical protein kinase C phosphorylates Par6 and facilitates transforming growth factor β-induced epithelial-to-mesenchymal transition.”. All experiments and figures in the chapter were completed by Adrian Gunaratne.

Portions of Chapter 1 and Chapter 5 were published in Cell Adhesion and Migration in July 2013 (Cell Adh Migr. 2013 Jul-Aug;7(4):357-61.)

This commentary is entitled “Par6 is phosphorylated by aPKC to facilitate EMT”. All figures and text in this invited article that were used in Chapters 1 and 5 of this thesis were completed by Adrian Gunaratne.

Chapter 4 of this thesis was prepared for submission

This paper is entitled “aPKC knockdown alters TGFβ response in NSCLC cells via both Smad-dependent and Smad-independent pathways”. Figure 4A and 8C were composed by an undergraduate student that I supervised, Mr. Tarek El-Chabib, when he carried out a thesis project in the Di Guglielmo laboratory (Western University, Canada). The quantitation of apoptotic nuclei in Figure 4.7A was also conducted by Tarek El-Chabib. These
experiments were repeated by Adrian Gunaratne. Microarray analysis was conducted by David Carter from the London Regional Genomics Centre (London, Canada) as outlined in the Methods section of this chapter. All other experiments and figures in this chapter were completed by Adrian Gunaratne.
Acknowledgments

Completing my Ph.D. at Western would not have been possible without the help, guidance, and support of many wonderful people.

Firstly, I would like to thank my supervisor Dr. John Di Guglielmo. Thank you for taking a chance on me and accepting me into your lab. The long, winding, stoney path of scientific discovery was always made bearable through your encouragement and support. In the time that I was here, your enthusiasm and passion inspired me not only in science, but also in life in general. It was in your lab that I learned how to complete my first western blot (you may not remember this, but you trained me personally). It was also in your lab that I learned that with the right attitude and perspective, one can make the best of any situation. This is a lesson I will take with me for the rest of my life…long after robotic droids have overtaken all SDS-PAGE gels. I am extremely grateful and feel profoundly lucky to have had you as a mentor and friend. I could not have asked for a better supervisor.

I would also like to thank the members of my advisory committee, Drs. Peter Chidiac and Jeff Dixon, and my past and present GSRs Drs. Jane Rylett and Marco Prado. Your thoughtful guidance and helpful discussions gave me direction throughout my Ph.D. studies.

This journey also was filled with many friends and colleagues that shared in all of the ups and downs that come with primary research. I would like to thank all the members (past and present) of the Di Guglielmo laboratory. I feel very blessed to have been part of the “lab family”. Thank you Ciric To, Eddie Chan, Sarah McLean, and Boun Thai for the many memories and laughs; Thank you also to the many friends we made in our “extended lab family” including the members of the Bhattacharya and Hammond Labs as well as all of the temporary students we had in the lab. Graduate school wouldn’t have been the same without all of you. I would also like to thank all of my friends outside of graduate school, including my old fest housemates Solomon Khan, Carolina Oteiza, and Pawel Przeracki who always provided support and laughs throughout the Ph.D. journey – despite not really understanding what I was up to. Sushi, fried chicken parties, enormous burgers and barbeques will all dominate many of my memories of graduate school. Thank you all for that.
Finally I would like to thank my family. Thank you to my brother Damian for always offering a shoulder to lean on during difficult times. Thank you to my mom and dad who instilled in my brother and I a desire for higher education, and then sacrificed their lives so we could attain it. You were both instrumental in inspiring my love of science and service. Thank you also to all of my other family members who have supported me always. Lastly, I would like to thank my wife Malki and my daughter Allison. Thank you both for understanding and putting up with the crazy life of a Ph.D. student. Knowing that I have such a wonderful, supporting, and loving family made everything so much easier.
Table of Contents

Abstract ............................................................................................................................... ii

Co-Authorship Statement ................................................................................................. iv

Acknowledgments ........................................................................................................... vi

Table of Contents ............................................................................................................ viii

List of Tables .................................................................................................................. xiv

List of Figures .................................................................................................................. xv

List of Abbreviations ...................................................................................................... xix

Chapter 1 .......................................................................................................................... 1

1 Introduction .................................................................................................................. 2

1.1 Introduction to the TGFβ pathway .......................................................................... 2

1.1.1 TGFβ ligands ...................................................................................................... 3

1.1.2 TGFβ receptors ................................................................................................. 4

1.1.3 Smads ................................................................................................................ 8

1.1.4 Pleiotropic effects of TGFβ ........................................................................... 11

1.2 Regulation of TGFβ signal transduction ................................................................. 14

1.2.1 Endocytosis and membrane trafficking ......................................................... 14

1.2.2 Classical clathrin-dependent endocytosis ....................................................... 15

1.2.3 Non-classical endocytosis: Membrane-rafts .................................................. 16

1.2.4 Endocytic trafficking regulates TGFβ receptor activity .................................. 17

1.2.5 Regulation of Smads ......................................................................................... 22

1.3 Protein Kinase C .................................................................................................... 25

1.3.1 The Protein Kinase C Family ........................................................................... 25

1.3.2 Protein Kinase C and Membrane Trafficking/Endocytosis ......................... 29

1.3.3 Atypical Protein Kinase C ............................................................................... 29
3.3.5 Scratch Assays and Polarization Assay .................................................. 116
3.3.6 Immunofluorescence Microscopy................................................................. 116
3.3.7 Transwell Migration Assays ........................................................................ 116
3.3.8 EMT and Migration ...................................................................................... 117
3.3.9 Reverse Transcription, Real time PCR and Statistical Analyses ............... 118
3.3.10 Site Directed Mutagenesis .......................................................................... 119
3.3.11 Statistical Analysis ...................................................................................... 119

3.4 Results ............................................................................................................. 119

3.4.1 TGFβ receptors and aPKC interact to the leading edge of migrating cells ............................................................................................................. 119
3.4.2 TGFβ receptors and PKC interact via Par6 ................................................. 123
3.4.3 aPKC phosphorylates Par6 ........................................................................... 125
3.4.4 aPKC phosphorylates Par6 independent of TGFβ receptors .................... 125
3.4.5 Par6 acts as a scaffold between aPKC and TGFβ receptors ..................... 131
3.4.6 aPKC expression and association increases Par6 levels .......................... 134
3.4.7 Par6 phosphorylation and aPKC association are important for Par6 induced migration ..................................................................................................... 137
3.4.8 aPKC siRNA alters RhoA levels ................................................................. 140
3.4.9 TGFβ treatment activates aPKC ................................................................. 142
3.4.10 aPKC siRNA induces changes in cell morphology ................................. 144
3.4.11 aPKC siRNA reduces TGFβ induced EMT ............................................. 147
3.4.12 aPKC knockdown reduces claudin loss and individual aPKC siRNA reduces TGFβ induced EMT ............................................................... 148
3.4.13 Phospho-mimetic Par6 induces TGFβ-dependent EMT and RhoA degradation in aPKC silenced cells................................................................. 153

3.5 Discussion .................................................................................................. 157

3.6 Footnotes ...................................................................................................... 159
3.7 References .................................................................................................................................................. 159

Chapter 4 .......................................................................................................................................................... 164

4 Chapter 4 .......................................................................................................................................................... 165

4.1 Chapter Summary .......................................................................................................................................... 165

4.2 Introduction .................................................................................................................................................... 166

4.3 Materials and Methods .................................................................................................................................. 168

4.3.1 Antibodies and Reagents .............................................................................................................................. 168

4.3.2 Cell Culture and Transfections ..................................................................................................................... 168

4.3.3 Protein Concentrations .................................................................................................................................. 169

4.3.4 Immunoblotting and Immunoprecipitation ................................................................................................. 169

4.3.5 Cellular Fractionation .................................................................................................................................... 169

4.3.6 Immunofluorescence Microscopy .................................................................................................................. 169

4.3.7 RNA Quality Assessment, Probe Preparation and GeneChip Hybridization .............................................. 170

4.3.8 Reverse Transcription, Real time PCR and Statistical Analyses ................................................................. 170

4.3.9 Cell Death Assays ......................................................................................................................................... 172

4.3.10 Statistical Analysis .................................................................................................................................... 172

4.4 Results ............................................................................................................................................................ 172

4.4.1 Knockdown of Atypical PKC isoforms alters TGFβ induced gene expression .............................................. 172

4.4.2 Knockdown of Atypical PKC alters TGFβ induced gene expression in qPCR analyses ............................... 178

4.4.3 aPKC knockdown reduces TGFβ induced Smad2 nuclear accumulation ..................................................... 182

4.4.4 siRNA targeting aPKC does not alter TNFα induced NF-κB nuclear translocation ...................................... 185

4.4.5 Knockdown of aPKC enhances P-p38 MAPK levels ..................................................................................... 187

4.4.6 Inhibition of p38 MAPK does not rescue Smad2 nuclear translocation. 190
4.4.7 Knockdown of aPKC increases TGFβ induced apoptotic response via p38 MAPK ................................................................. 193

4.4.8 Knockdown of aPKC also mediates TGFβ effects in H1299 NSCLC cells ................................................................. 193

4.4.9 Knockdown of aPKC increases TβRI-TRAF6 complexes ............... 199

4.4.10 Knockdown of TRAF6 in aPKC silenced cells reduces activated p38 MAPK and apoptosis ........................................ 201

4.4.11 Knockdown of aPKC reduces Smad2-Smad4 complex formation....... 204

4.4.12 Knockdown of aPKC increases SARA expression ...................... 207

4.4.13 Knockdown of aPKC increases cytosolic retention of Smad2 by SARA ........................................................................... 207

4.5 Discussion ................................................................................................................................. 214

4.6 Footnotes ........................................................................................................................................ 217

4.7 References ................................................................................................................................... 217

Chapter 5 ........................................................................................................................................ 223

5 Chapter 5 ....................................................................................................................................... 224

5.1 General Summary ...................................................................................................................... 224

5.2 General Discussion and Future Directions .............................................................................. 227

5.2.1 Regulation of TGFβ receptor trafficking and signalling by atypical protein kinase C ...................................................... 227

5.2.2 Atypical Protein kinase C phosphorylates Par6 to facilitate EMT ....... 229

5.3 Limitations and Future Directions .......................................................................................... 236

5.4 Conclusions ............................................................................................................................... 241

5.5 References ............................................................................................................................... 242

Appendix ........................................................................................................................................ 247

Curriculum Vitae ............................................................................................................................ 248
List of Tables

Table 4.1. aPKC knockdown alters TGFβ gene response by microarray analysis............. 177
List of Figures

Figure 1.1. TGFβ superfamily signalling components .......................................................... 7
Figure 1.2. Classical TGFβ receptor activation and signalling ........................................... 10
Figure 1.3. The multi-functional nature of TGFβ................................................................. 13
Figure 1.4. Endocytosis and trafficking control TGFβ receptor activity .............................. 21
Figure 1.5. Schematic representation of the PKC family .................................................... 28
Figure 1.6. Potential roles for aPKC in TGFβ pathways .................................................... 33
Figure 1.7. TGFβ receptors phosphorylate Par6 to trigger EMT ......................................... 38
Figure 1.8. Features of EMT ................................................................................................. 43
Figure 1.9. TGFβ can activate MAPK pathways independently of Smads. ....................... 46
Figure 1.10. Exploring a role for aPKC in Smad-dependent and Smad-independent pathways ......................................................................................................................... 50
Figure 2.1. PKC inhibition alters TGFβ receptor trafficking .............................................. 81
Figure 2.2. PKC inhibition extends TGFβ receptor half-life .............................................. 84
Figure 2.3. PKC inhibition extends TGFβ induced Smad2 phosphorylation ....................... 87
Figure 2.4. aPKC inhibition extends TGFβ induced Smad2 phosphorylation .................... 90
Figure 2.5. aPKC overexpression reduces steady state TGFβ receptor levels ..................... 93
Figure 2.6. PKC inhibition reduces clearance of ubiquitinated TGFβ receptor complexes .. 96
Figure 2.7. aPKC knockdown reduces TGFβ receptor degradation .................................... 99
Figure 2.8. aPKC knockdown extends Smad2 phosphorylation ....................................... 101
Figure 2.9. aPKC knockdown extends TGFβ induced Smad2 phosphorylation even after 24 hours

Figure 2.10. Regulation of TGFβ receptor trafficking and signalling by atypical protein kinase C

Figure 3.1. Atypical PKCδ co-localizes with TGFβ receptors at the leading edge of migrating cells

Figure 3.2. Atypical PKCδ associates with TGFβ receptors through Par6

Figure 3.3. aPKCs associate and phosphorylate Par6 on S345

Figure 3.4. aPKC can phosphorylate Par6 independent of TGFβ receptors

Figure 3.5. aPKC reduces TβRI associated Par6

Figure 3.6. aPKC expression stabilizes Par6 protein levels

Figure 3.7. Par6 induces cell migration

Figure 3.8. aPKC silencing attenuates TGFβ-dependent decreases in RhoA levels

Figure 3.9. TGFβ increases total and Par6 associated aPKC activity

Figure 3.10. aPKC silencing reduces TGFβ induced changes in cell morphology

Figure 3.11. aPKC silencing reduces TGFβ induced EMT and motility

Figure 3.12. aPKC silencing reduces TGFβ induced Claudin-1 loss and individual aPKC knockdown also reduces TGFβ induced E-cadherin loss

Figure 3.13. A S345E phospho-mimetic Par6 mutant restores TGFβ-dependent E-cadherin and RhoA loss in aPKC silenced cells

Figure 3.14 Atypical PKC phosphorylates Par6 and facilitates TGFβ induced EMT

Figure 4.1. aPKC knockdown prolongs TGFβ induced Smad2 phosphorylation
Figure 4.2. aPKC silencing alters TGFβ dependent gene induction............................ 181
Figure 4.3. aPKC knockdown reduces TGFβ induced Smad2 nuclear accumulation....... 184
Figure 4.4. aPKC knockdown does not alter TNFα induced NF-κB nuclear accumulation 186
Figure 4.5. aPKC knockdown increases and prolongs p38 MAPK phosphorylation in response to TGFβ.............................................................. 189
Figure 4.6. p38 MAPK inhibition of aPKC knockdown cells does not rescue Smad2 nuclear accumulation.......................................................................................... 192
Figure 4.7. aPKC knockdown enhances TGFβ induced apoptotic response............... 196
Figure 4.8. aPKC knockdown in H1299 cells also reduces Smad2 nuclear accumulation, increases p38 MAPK levels, and enhances apoptosis, similar to A549 cells......... 198
Figure 4.9. aPKC knockdown increases levels of TβRI-TRAF6 complexes .............. 200
Figure 4.10. Knockdown of TRAF6 abrogates TGFβ-p38 MAPK effects observed in aPKC depleted cells.............................................................................................................. 203
Figure 4.11. aPKC knockdown reduces TGFβ-induced Smad2-Smad4 complex formation206
Figure 4.12. Knockdown of aPKC increases steady state SARA protein levels ......... 210
Figure 4.13. Knockdown of aPKC reduces TGFβ induced Smad2 release from SARA .... 212
Figure 4.14. aPKC knockdown alters TGFβ induces Smad-dependent and Smad-independent pathways ............................................................................................................ 213
Figure 5.1. aPKC affects TGFβ signalling pathways ...................................................... 226
Figure 5.2. Roles for Par6 phosphorylation in EMT and Migration............................ 235
Figure 5.3. aPKC may control the subcellular itinerary of activated TGFβ receptor complexes to control specific TGFβ signalling outcomes......................................................... 240
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>A</td>
<td>Alanine</td>
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<tr>
<td>AJ</td>
<td>Adherens junctions</td>
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<tr>
<td>Act-RI</td>
<td>Activin receptor type I</td>
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<td>Act-RIB</td>
<td>Activin receptor type IB</td>
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<td>Act-RII</td>
<td>Activin receptor type II</td>
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<td>Act-RIIB</td>
<td>Activin A receptor type IIB</td>
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<tr>
<td>ALK</td>
<td>Activin receptor-like kinase</td>
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<tr>
<td>AMH</td>
<td>Anti müllerian hormone</td>
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<td>AMHR-II</td>
<td>Anti müllerian hormone receptor type II</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AP-2</td>
<td>Adaptor protein 2</td>
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<tr>
<td>aPKC</td>
<td>Atypical Protein Kinase C</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>b-TGFβ</td>
<td>Biotinylated transforming growth factor beta</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>Cyclin dependent kinase 6</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>Death inducing signalling complex</td>
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<td>Dulbecco’s modified eagle medium</td>
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<td>E-cad</td>
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<td>ECM</td>
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<td>Early endosomal antigen-1</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>Epidermal growth factor receptor substrate 15</td>
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<td>ERK</td>
<td>Extracellular signal-related kinase</td>
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<td>FYVE</td>
<td>Fab1, YOTB, Vac1, EEA1</td>
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<td>GAPDH</td>
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<td>GDF</td>
<td>Growth and differentiation factor</td>
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<td>G protein coupled receptor</td>
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<td>Glycogen synthase kinase 3 beta</td>
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<td>h</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>HECT</td>
<td>homologous to the E6-AP carboxyl terminus</td>
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</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>NPC</td>
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<td>Prostate apoptosis response 4</td>
</tr>
<tr>
<td>Par6</td>
<td>Partitioning defective 6</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDZ</td>
<td>Post synaptic density protein drosophila large disc tumor suppressor zona-occludens 1 protein</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
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<td>Protein kinase C alpha</td>
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<td>PMSF</td>
<td>Phenylmethanesulfonylefluoride</td>
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<tr>
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<td>Polymerase RNA II DNA directed polypeptide A</td>
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<td>Phosphorylated Smad</td>
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<td>quantitative polymerase chain reaction</td>
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<td>Receptor regulated smad</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>RhoA</td>
<td>Ras homolog gene family member A</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>SARA</td>
<td>Smad anchor for receptor activation</td>
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<tr>
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<td>Serine</td>
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<tr>
<td>S345A</td>
<td>Serine 345 mutated to alanine</td>
</tr>
<tr>
<td>S345E</td>
<td>Serine 345 mutated to glutamic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>Serine</td>
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<td>Shc</td>
<td>Src homology 2 domain containing</td>
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<td>Small interfering RNA</td>
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<td>SMA</td>
<td>Smooth muscle actin</td>
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<td>Small phenotype mothers against decapentaplegic homolog</td>
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<tr>
<td>Smurf2</td>
<td>Smad ubiquitin regulatory factor-2</td>
</tr>
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<td>Snail family zinc finger 1 (Snail)</td>
</tr>
<tr>
<td>SNAI2</td>
<td>Snail family zinc finger 2 (Slug)</td>
</tr>
<tr>
<td>Sos</td>
<td>Sons of sevenless</td>
</tr>
<tr>
<td>Abbreviation</td>
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</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGFβ-activated kinase 1</td>
</tr>
<tr>
<td>TβRI</td>
<td>Transforming growth factor beta receptor I</td>
</tr>
<tr>
<td>TβRII</td>
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<tr>
<td>TβRIII</td>
<td>Transforming growth factor beta receptor III</td>
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<td>Tris buffered saline with tween</td>
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<td>TIEG1</td>
<td>TGFβ inducible early gene response 1</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
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<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
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<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>TNFR1</td>
<td>Tumour necrosis factor receptor 1</td>
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<td>Tumor necrosis factor receptor associated factor 6</td>
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<td>Ubiquitin</td>
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Chapter 1

INTRODUCTION

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1 Introduction

1.1 Introduction to the TGFβ pathway

The transforming growth factor beta (TGFβ) signalling pathway represents a class of molecules that are essential regulators of embryonic development and tissue homeostasis. TGFβ regulates diverse cellular processes including cell differentiation, proliferation, migration, apoptosis, and cell plasticity.

Given the importance of TGFβ signalling in tissue homeostasis, aberrant signalling leads to various pathologies and disorders including diseases of immunity and inflammation, cancer, and fibrosis. Paradoxically, TGFβ can both inhibit, and promote cancer, as it acts as a tumour suppressor in the early stages of cancer development, but a tumour promoter in late stage cancers.

It has been over 30 years since TGFβ was first discovered as a multifunctional cytokine[1], and although the general pathways surrounding TGFβ have been identified, it has long been known that the contextual nature of TGFβ hints at a pathway more complicated than the sum of its seemingly simple parts. Since its discovery, researchers have uncovered that the pleiotropic nature of TGFβ is controlled by a network of regulators along various points in the pathway that control the activity and outcome of TGFβ signalling; In turn, how this pathway and its various components are regulated, can dictate how cells respond to TGFβ signals.

Thus, knowledge of the mechanisms that govern the regulation and the signalling of this incredibly powerful system are important to increase our understanding of animal embryo development, tissue homeostasis, and diseases such as cancer.
1.1.1 TGFβ ligands

The TGFβ superfamily, which includes over 30 cytokine members, are involved with the binding and the activation of ser/thr kinase receptors to mediate signalling cascades and transcriptional events that control cellular homeostasis and metazoan development (reviewed in [2-7]). These structurally related cytokines are characterized by 6 conserved cysteine residues and the genes are encoded by 42 open reading frames in the human genome [8]. The superfamily is further divided into 2 major subfamilies based on sequence similarities and the downstream pathways they activate: specifically the TGFβ/Activin/Nodal subfamily and the BMP/GDF/MIS (Bone morphogenetic protein, growth and differentiation factor, Muellerian inhibiting substance) subfamily [9].

TGFβ was the first discovered member of the superfamily, and is often considered the prototype. There are three TGFβ isoforms in mammals: TGFβ1, TGFβ2 and TGFβ3. Each isoform is encoded from a different gene, although all activate the same receptor system [6]. TGFβ1 is the major isoform in adults, as its expression is the most predominant and ubiquitous, although all three isoforms are expressed during development [10, 11]. Of the three ligands TGFβ1 is also the most frequently upregulated in tumor cells and is often the focus of TGFβ studies in tumorigenesis [3, 12, 13].

TGFβ ligands are homo-dimeric proteins with each monomer forming several extended β-strands that interlock through three intrachain disulfide bonds. These disulfide bridges form a tight structure common to TGFβ ligands known as the “cysteine knot” [14]. The dimer is stabilized through hydrophobic interactions and also by an inter-subunit disulfide bond [14].

TGFβ ligand is secreted as an inactive latent complex with two pro-segment peptides [15]. The pro-peptide is called the latency-associated protein (LAP) and TGFβ bound to LAP is not recognized by the TGFβ receptors. LAP also links TGFβ homodimers to latent TGFβ-binding proteins (LTBPs) through direct disulfide bonds to LAP. LTBPs can target the TGFβ-LAP complex to the cell surface for activation or to the extracellular matrix for storage [16-18]. This latency complex mechanism safeguards against inadvertent activation and allows for sequestration in the extracellular matrix.
Importantly, sequestration of ligand in the ECM allows for a reservoir of readily available ligand without the need for synthesis [17, 18].

The regulated activation process of TGFβ involves preferential cleavage and degradation of the TGFβ prosegments by activating molecules and proteases. Latent TGFβ can be activated by proteases and ECM proteins such as matrix metalloprotease 2 (MMP2), matrix metalloprotease 9 (MMP9), plasmin and thrombospondin [19-22]. It is important to note that these proteases are often expressed in malignant cells and are highly active at sites of cell migration and invasion – giving indication that the activation of TGFβ plays a role in these processes [21, 22].

Once TGFβ ligand is activated, it mediates downstream transcriptional events through the binding and activation of the TGFβ receptors.

1.1.2 TGFβ receptors

TGFβ ligands bind and activate a family of transmembrane proteins known as the TGFβ receptors. There are two major TGFβ receptor kinase subtypes in the superfamily categorized by their structural and functional properties: type I receptors and type II receptors. Type I and type II receptors are structurally related glycoproteins consisting of a N-terminal extracellular ligand binding domain, a transmembrane domain, and a ser/thr kinase domain near the C-terminus [6, 23, 24]. Together, these receptors mediate intracellular signals upon activation and are crucial to the mediation of TGFβ stimulated responses.

There are seven type I and five type II receptors in the human genome, all of which mediate TGFβ signalling [25] (Figure 1.1). The type II receptors consist of: ActRII, ActRIIB, TβRII, BMPRII and AMHRII [6]. The seven type I receptors were originally systematically classified as the activin receptor-like kinases (ALK), thus named ALK1-7. As the physiological ligands of the ALK receptors became known, more descriptive names were introduced [6]. For example, the Type I TGFβ receptor (TβRI) in the prototypical TGFβ pathway is also known as ALK5 [26, 27].
At the cell surface, type II receptors form dimers that bind TGFβ ligand. Ligand binding promotes the binding of type I receptor dimers, thus forming an activated ligand/heterotetrameric receptor signalling complex [6, 7]. Classical TGFβ signalling involves type II receptors (TβRII) phosphorylating TβRI, thereby activating it. Active TβRI in turn phosphorylates intracellular signalling mediators such as Smads, which regulate the transcription of TGFβ target genes to mediate cellular response (Figure 1.2) [23].

TβRII is a constitutively active ser/thr protein kinase which is found on the cell surface as a homodimer in the presence or absence of ligand. TGFβ ligand enhances the formation of heteromeric complexes of TβRII homodimers and TβRI homodimers (reviewed in [2, 28]). Interestingly, overexpression of TβRI and TβRII can also promote the formation of the heteromeric complex without the addition of ligand [29]. Once TβRI and TβRII are in a heteromeric complex, the constitutively active TβRII transphosphorylates TβRI on a unique glycine-serine sequence termed the “GS domain”. The GS domain is a thirty amino acid region adjacent to the kinase domain of TβRI containing a characteristic SGSGSG sequence, and is critical for proper TGFβ signalling [23]. In the basal state, the GS domain of TβRI presses against the catalytic center of the kinase domain – thereby holding TβRI inactive [30]. TβRII phosphorylation of the GS domain triggers the activation of TβRI [30]. The phosphorylated GS domain also acts as a docking site for “Smads” the canonical intracellular mediators of TGFβ signals and mutations of serine and or glycine residues in the TβRI GS domain can impair TGFβ signalling potential [31]. In addition to the unique GS domain, TβRI and TβRII have several other key differences. TβRI has a shorter extracellular domain than TβRII and does not bind ligand independently [2, 32]. TβRII also possesses a ser/thr rich intracellular tail, which is not found in TβRI [28]. Furthermore, unlike TβRII, TβRI kinase activity is not constitutively active. However, mutational studies have discovered that mutation of threonine 204 to aspartic acid locks the kinase in an active state and can transduce TGFβ signals in the absence of ligand [31].

A third receptor type, termed the type III receptor, assists in TGFβ signalling by facilitating the presentation of ligand to the type II receptor [33, 34]. There are two type
III receptors, known as endoglin, and betaglycan (also referred to as TβRIII). These membrane anchored receptors do not have enzymatic activity and are considered accessory receptors as TGFβ signals can still be propagated in their absence [6]. Betaglycan can bind all three TGFβ ligand isoforms with high affinity [35, 36], but is most evidently important with TGFβ2 ligand, which has a low intrinsic affinity for binding TβRII in the absence of betaglycan [36]. Unlike betaglycan, which shows a greater range of expression, endoglin is primarily expressed in endothelial cells, and interestingly, does not bind TGFβ2 [37-39]. Although, thought of as accessory receptors that can aid in signalling, type III receptors can also inhibit TGFβ signalling potential. For example, betaglycan’s extracellular domain can be released from the membrane; this soluble betaglycan can thereby sequester TGFβ ligand and inhibit TGFβ signalling [40]. Thus, the exact role of type III receptors is complicated as they can both enhance and reduce the signalling potential of TGFβ isoforms in varying contexts.
Figure 1.1. TGFβ superfamily signalling components

The core components of various TGFβ pathways (vertebrate) are shown in this diagram. TGFβ ligands bind type II receptors, which bind and phosphorylate type I receptors. Type I receptors phosphorylate corresponding R-Smads, which bind the common Smad (Smad 4) to form a transcriptional complex. I-Smads can inhibit R-Smad signalling by competitively binding to type I receptors thereby excluding R-Smads from phosphorylation. Shown in bold is the TGFβ pathway studied in this thesis.
1.1.3 Smads

The Smad family of proteins are the originally identified substrates of the type I receptor family and are essential in the co-ordination and regulation of the TGFβ signal transduction process. The Smads were first identified as the gene products of the *Drosophila* MAD gene (Mothers against Decapentaplegic [DPP]) and the *C. elegans* Sma gene [41-43]. Smads are ubiquitously expressed during development and in all adult tissues [44, 45] and the deregulation of Smads are associated with numerous cancers (reviewed in [12, 46]). Smads have two highly conserved “Mad Homology” (MH) domains: an N-terminal MH1 domain and a C-terminal MH2 domain, which are attached by a proline rich “linker” region [6, 23].

There are eight mammalian Smads divided into three functional groups: the receptor regulated Smads (R-Smad), the common-Smad and the inhibitory Smads (I-Smad) (Figure 1.1). Upon receptor activation, the type I receptor binds and phosphorylates R-Smads, which then oligomerize with the co-Smad to regulate transcriptional events at the nucleus. Alternatively, I-Smads can bind the activated receptor, thereby blocking R-Smad binding, and subsequently recruiting ubiquitin ligases to target the receptor complex for degradation.

The receptor regulated Smads (R-Smads) consists of Smad1, Smad2, Smad3, Smad5 and Smad8. Smad2 and Smad3 are involved in the TGFβ/Activin pathways, whereas Smad1, Smad5 and Smad8 participate in the BMP signalling pathway (Figure 1.1) [2, 23].

The specificity of the various R-Smad and type I receptor interactions is mediated by the “L45 loop” in the kinase region of type I receptor and the L3 loop in the MH2 domains of R-Smads [47]. In the classical TGFβ pathway, the type I receptor is TβRI, the primary R-Smad is Smad2, and the I-Smad is Smad7. The phosphorylated GS region of activated TβRI acts as a docking site for Smad2. This interaction occurs via a positively charged surface patch present in the Smad2 MH2 domain [48, 49] and mutations in this basic patch of Smad2 limit its binding and activation by TβRI [50].

R-Smads are directly activated by type I receptors through phosphorylation. R-Smads contain an evolutionarily conserved SSXS motif in their MH2 domain, and the
two most C-terminal serine (S) residues become directly phosphorylated by type I receptors [6, 23, 51]. Phosphorylated Smad2 dissociates from TβRI, and the phosphorylated SXS region of Smad2 binds to a positively charged surface pocket on the MH2 domain of the co-Smad, Smad4 [23]. The R-Smad-Smad4 heteromeric complex then translocates to the nucleus where it binds to promoters of TGFβ target genes, or interacts with various co-activators or co-repressors that ultimately control TGFβ specific transcriptional programs [6, 7].

Unlike the R-Smads and co-Smads, which carry TGFβ signals to the nucleus, I-Smads (Smad 6 and Smad7) act antagonistically to reduce TGFβ signal transduction [6, 7]. Smad7 primarily functions in the TGFβ/Activin and BMP pathways, whereas Smad6 preferentially functions in the BMP pathway. The MH1 domains of I-Smads show little similarity to the MH1 domains of the R-Smads, but the MH2 domains of R-Smads, co-Smad and I-Smads are homologous. However, unlike the R-Smads, the I-Smads do not contain the characteristic C-terminal SSXS phosphorylation sequence. Lacking the ability to be phosphorylated, this allows for I-Smads to stably interact with an activated receptor, thereby antagonizing signalling by competing with R-Smads for activated receptor binding [6, 7]. Furthermore, I-Smads mediate the interaction between the receptors and Smurf 1 and Smurf2 (Smad ubiquitination regulatory factors), which are E3 ubiquitin ligases that ubiquitinate and target the receptors for degradation, thereby suppressing further TGFβ signalling [52, 53].
Figure 1.2. Classical TGFβ receptor activation and signalling

TGFβ signalling is initiated at the cell surface when the TGFβ type II receptor (TβRII) binds TGFβ ligand. Ligand binding promotes the binding of the TGFβ type I receptor (TβRI), leading to the formation of an activated ligand/heterotetrameric receptor signalling complex. TβRII activates TβRI by phosphorylating it on its GS domain. The active type I receptor in turn phosphorylates intracellular signalling mediators such as Smad2, which stimulates the binding of Smad4 to the R-Smad. Smad2-Smad4 association creates a transcriptional unit that accumulates in the nucleus to regulate the transcription of TGFβ target genes. The activated TGFβ receptors can also activate various Non-Smad signalling pathways.
1.1.4 Pleiotropic effects of TGFβ

The effects exerted by TGFβ signalling have long been known to be cell and context dependent (reviewed in [5, 12, 54]. In mature tissues, TGFβ has multiple roles that ultimately lead to homeostasis. For example, TGFβ stimulates fibroblasts of the stroma to grow and deposit extra cellular matrix proteins; TGFβ inhibits the growth of endothelial cells and controls their morphogenesis; TGFβ can inhibit cells of the immune system by inhibiting T-cell proliferation and natural killer cell function; and in the epithelium, TGFβ controls cell growth through stimulating cell cycle arrest, apoptosis, and adhesion (Figure 1.3) (reviewed in [12]). Given the important homeostatic role for TGFβ signalling, its deregulation leads to various pathologies. For example, aberrant TGFβ signalling is a hallmark of many epithelial derived cancers [3, 55], and drives tumour progression. Normally, TGFβ acts as a tumour suppressor by controlling the growth of the epithelium, but in many tumours TGFβ undergoes a role switch and becomes a cancer promoting metastatic agent [3, 4, 55-58]. The growth suppressive function of TGFβ was originally described to be through the phosphorylation of RB (retinoblastoma protein) [59]. RB functions as a cell cycle gatekeeper, and in its underphosphorylated state, prevents cells from entering into mitosis from the G1 checkpoint of the cell cycle [60]. Interestingly, TGFβ arrests cells in the G1 phase of the cell cycle through the prevention of RB phosphorylation thereby leading to growth suppression [59]. Phosphorylation of RB occurs through cyclin dependent kinases CDK4 and CDK6 [60]. TGFβ stimulation leads to the upregulation of the CDK inhibitors p15 and p21, which prevent the actions of CDK4 and CDK6 thereby preventing RB phosphorylation and the ultimately stimulating growth arrest [5, 12]. Other mechanisms of growth arrest include the TGFβ induced repression of growth stimulatory transcription factors including Myc, and members of the ID family [5, 12, 54]. Thus, although the mechanisms can differ among various cell types, TGFβ normally causes growth arrest in mature epithelium, maintaining it in a homeostatic state.

In addition to controlling epithelial cells through a cytostatic program, TGFβ can also stimulate apoptosis to maintain homeostasis. Apoptosis is a form of programmed cell death and is important for normal cell turnover; however, deregulation of apoptosis is
also involved in various pathological disorders (reviewed in [61]). Apoptotic signalling can be classified into extrinsic (via death receptor), and the intrinsic (mitochondrial) pathways, however, each pathway can influence the other. Activation of either pathway leads to the cleavage of a group of cysteine proteases called caspases, which execute the apoptotic program to result in DNA fragmentation, chromatin condensation, protein degradation, and membrane blebbing, which leads to the formation of cell fragments called apoptotic bodies. These apoptotic bodies are subsequently engulfed by phagocytic cells such as macrophages [61]. Because apoptotic bodies do not release the cellular contents into the surrounding interstitial tissue, an inflammatory response is not triggered [61]. The extrinsic pathways that trigger apoptosis utilize transmembrane death receptors which most often include members of the tumor necrosis factor (TNF) receptor gene superfamily [62]. The best characterized of these are the Fas ligand/Fas receptor complex and the TNF-α/TNFRI models. Activation of these receptors by their ligands leads to the recruitment of cytoplasmic adaptor proteins to form a death-inducing signalling complex (DISC) [63]. DISC ultimately triggers the activation of caspases through cleavage [61]. The intrinsic apoptosis pathway involves non-receptor mediated signals that cause changes in the inner mitochondrial membrane that disrupt membrane potential resulting in the release of normally sequestered pro-apoptotic proteins into the cytosol [61].

TGFβ has been reported to operate in both apoptotic pathways. For example, TβRII can interact directly with the pro-apoptotic adapter protein DAXX, a member of the Fas pathway, which leads to the activation of JNK and the induction of apoptosis of epithelial cells [64]. Furthermore, TGFβ can also operate in the intrinsic pathway through its transcriptional induction of DAPK (death associated protein kinase) [65]. DAPK can regulate cytochrome C release from mitochondria, and ultimately apoptotic response [65].

Thus, the cytostatic and apoptotic actions of TGFβ signalling make it a clear regulator of cellular and tissue homeostasis. Deregulation of various components of the TGFβ pathway often lead to diseases of hyperproliferation, such as cancer.
TGFβ signalling has cell type and context-specific effects. TGFβ has an important role in controlling the growth homeostasis of the epithelium by regulating cell-cycle arrest and apoptosis. Whereas in fibroblasts, TGFβ stimulates ECM production and proliferation, important aspects of tissue repair. However, as tumours progress, the growth inhibitory aspects of TGFβ are lost, and then TGFβ signalling can stimulate EMT, migration, and invasion, thereby facilitating tumour progression.
1.2 Regulation of TGFβ signal transduction

It has become clear that the complex nature of TGFβ responses is partly related to the regulation of TGFβ receptor signalling. The importance of this regulation is highlighted by the complex roles of TGFβ in development, and the detrimental consequences of aberrant TGFβ signalling in various diseases such as cancer and fibrosis. Much work has been devoted to understanding the mechanisms that govern the regulation of TGFβ receptor signalling, however, many questions still remain to be addressed.

1.2.1 Endocytosis and membrane trafficking

Endocytosis of cell surface receptors has been shown to be an important regulatory event. Endocytosis refers to the process by which membrane associated molecules are taken into the cellular environment through internal membrane compartments (reviewed in [66]). This process involves the invagination of the plasma membrane to encapsulate the cargo, followed by budding from the membrane, and the formation of an intracellular vesicle containing the internalized molecules. Importantly, endocytosis of cell surface receptors can modulate signalling processes by spatially removing receptors from accessing extracellular ligand, but also opens complex avenues of signalling or down-regulation pathways depending on the intracellular itinerary of the cargo. Thus, how receptors are trafficked in the cell, can determine whether receptors are down-regulated or whether they can continue signalling.

Interestingly, modulation of TGFβ receptor activity is controlled by the endocytosis and trafficking of the TGFβ receptors. Cell surface TGFβ receptors are dynamic and are constitutively being internalized via “clathrin dependent” and “membrane raft- dependent” endocytosis. Furthermore, clathrin-dependent endocytosis positively influences signalling and propagates TGFβ Smad signalling, whereas membrane raft-dependent endocytosis results in receptor degradation and signal termination [67, 68].
1.2.2 Classical clathrin-dependent endocytosis

Clathrin-dependent endocytosis is a conserved mechanism which is responsible for the cellular internalization of pathogens, antigens, nutrients, growth factors and many receptor types (reviewed in: [66, 67, 69]). The defining feature of this endocytic pathway involves the recruitment of soluble clathrin from the cytoplasm to the plasma membrane. The clathrin triskelia aggregate at the membrane and create a polygonal lattice to form an invagination referred to as a “clathrin-coated pit”. Clathrin binding adaptor proteins, such as adaptor protein 2 (AP-2), promote the clathrin polymerization as well as bind other cargo proteins to facilitate their endocytosis. AP-2 as well as Eps15 (epidermal growth factor substrate 15) facilitates the formation of the clathrin lattice, leading to an increase in the plasma membrane curvature and creating the pit. The clathrin-coated pit pinches off from the plasma membrane to form a “clathrin-coated vesicle”. This process is dependent on Dynamin, a GTPase that promotes the scission of the clathrin coated pit into a newly formed intracellular vesicle. Following endocytosis, the newly formed vesicles are uncoated, and then become ‘early endosomes’. The early endosome is an important intracellular compartment that acts as a key sorting facility for the proteins it contains. The vesicular cargo can be sorted into recycling endosomes, which return to the cell surface, or can be trafficked to late endosomes where they are furthered targeted to the lysosome for degradation. Interestingly, these sorting and trafficking events can be directed by post-translational modifications of the cargo, such as ubiquitination, phosphorylation, or the interaction with specific proteins [69]. Furthermore, these directed trafficking events are controlled by a large family of small GTPases called the Rab GTPases. Rab GTPases are Ras-like small G-proteins that are tightly associated with membranes, molecular motors, and vesicular coat components and their activity controls endocytosis, trafficking, and exocytosis by regulating the processes of docking, fusion, and tethering between vesicular compartments [66, 67]. The different endosomal compartments are associated with distinct Rab GTPase function, and specific Rabs are often experimentally used as markers of specific compartments. For example, Rab4 and Rab5 are localized and enriched in the early endosome, Rab 4 and Rab 11 are enriched in recycling endosomes, and Rab7 and Rab 9 are enriched in late endosomes [66].
As with specific Rabs, the early endosome is also characterized by specific early endosome proteins containing a unique “FYVE” domain (Fab1, YOTB, Vac1, EEA1). The FYVE domain binds these proteins to the membrane lipid phosphatidylinositol-3-phosphate (PtdIns3P), which is highly enriched in the early endosome. FYVE domain proteins are thought to facilitate endosomal trafficking dynamics. For example, the early endosome marker EEA1 (early endosome antigen-1), is a FYVE domain containing effector of Rab5 that helps control early endosomal fusion [66, 67]. Thus, protein-protein interactions, as well as various protein-lipid interactions within endosomal compartments are important in guiding the sorting of intracellular cargo following clathrin-mediated endocytosis.

1.2.3 Non-classical endocytosis: Membrane-rafts

Clathrin-independent endocytosis is also a common mode of internalization for a number of molecules from the extracellular environment. Clathrin-independent endocytosis routes are sensitive to cholesterol depletion, pointing to the importance of membrane rafts (sometimes also called lipid rafts). Membrane rafts are liquid ordered membrane microdomains that are discrete from clathrin-coated pits. They are characterized by portions of the plasma membrane that have a very distinct lipid composition: they are rich in cholesterol, glycosphingolipids, and glycosylphosphatidylinositol-anchored (GPI) proteins [67, 70]. This unique composition makes membrane rafts less fluid and more rigid than non-raft areas of the plasma membrane [71]. Interestingly, these properties allow membrane rafts to create platforms that recruit and/or exclude specific lipids and proteins and thus can segregate cell surface components from the rest of the plasma membrane, allowing for another mechanism of endocytosis and protein trafficking [67, 71]. Interestingly, membrane raft-mediated endocytosis seems to be especially important for proteins that contain a GPI anchor [72, 73]. The spingolipids and cholesterol in membrane rafts tether GPI-anchored proteins by binding to the acyl chain on the GPI-anchor [74]. Another mechanism linking cell surface molecules to membrane rafts are through interactions with raft resident proteins such as caveolin, flotillin and annexin [67, 70, 73, 74].
Membrane rafts important to the regulation of the TGFβ pathway are “caveolae”. Caveolae are a subset of membrane rafts that are morphologically identifiable as flask-like invaginations of the plasma membrane approximately 60-80 nm in diameter [66, 70, 72, 75]. Caveolae, like membrane rafts, are rich in cholesterol and sphingolipids, but they are also highly enriched in a type of protein called the caveolins [76]. There are three types of caveolins: caveolin-1, caveolin-2, and caveolin-3. Caveolin-2 is only found expressed in muscle cells, whereas caveolin-1 and 2 show widespread cellular expression. However, caveolin-1 (cav-1) is the most important for the formation of caveolae in most cells and cav-1 knockout mice lack caveolar structures [77, 78]. Caveolae form through the oligomerization of cav-1 proteins and their association with cholesterol molecules [67, 79]. Similar to clathrin-mediated endocytosis, protein-protein interactions with constituents of membrane rafts, such as cav-1, can regulate entry into caveolae and control the endocytosis of molecules into these intracellular compartments. Caveolae-dependent endocytosis has been shown to be important for the internalization of various nutrients, viruses and cell surface receptors, including various GPCRs, RTKs and TGFβ receptors [67-70, 79, 80].

1.2.4 Endocytic trafficking regulates TGFβ receptor activity

Interestingly, TGFβ induced signalling events are intricately linked to the trafficking of TGFβ receptors (Figure 1.4) [68, 81-86]. TGFβ receptors are constitutively internalized via both membrane raft-dependent and clathrin-dependent endocytosis, and the specific trafficking of the TGFβ receptors, was determined to dictate whether the TGFβ signal would be propagated or degraded [68]. In this study, Di Guglielmo et al. report that the inhibition of clathrin-dependent endocytosis using Eps15 or Dynamin dominant negative mutants blocked the ability of TGFβ receptors to access the early endosome, and increased their propensity to access caveolae. Similarly, using a cholesterol depletion technique, which inhibits membrane-raft dependent endocytosis, receptors were less likely to access caveolae and found more in the early endosome. This interesting finding indicated that TGFβ receptors could internalize from the cell surface
at least two ways: \textit{via} clathrin coated pits into the early endosome or \textit{via} membrane rafts into caveolae – and these processes could be manipulated by altering trafficking machinery. It is interesting to note that because the TGF\(\beta\) receptors are constitutively being internalized, ligands in this system do not regulate trafficking events, but instead, stabilize heterotetrameric interactions between the Type I and II TGF\(\beta\) receptors [68].

Internalization of activated receptor complexes into distinct endocytic compartments, such as the early endosome or caveolae, serves to bring the receptors to distinct Smads and Smad associated proteins associated with each particular compartment. Moreover, the fate of the receptors and ultimately the TGF\(\beta\) signal, was related to the subcellular compartment that the receptors accessed. Receptors accessing the early endosome were more likely to phosphorylate Smad2 and propagate TGF\(\beta\) signalling, whereas receptors accessing caveolae were more likely to be targeted for degradation (Figure 1.4) [68]. This finding was particularly interesting, as classically the internalization of various membrane receptors into the early endosome was thought to solely be a mechanism by which signalling is reduced or for the down regulation of receptors. However, it is now known that endosomes can be highly specific signalling platforms for various receptors (reviewed in [69]) and this was true of the TGF\(\beta\) receptors.

Di Guglielmo et al. found that TGF\(\beta\) receptors that internalized into the early endosome \textit{via} clathrin promoted TGF\(\beta\) signalling through the enhanced activation of Smad2 [68]. TGF\(\beta\) receptors are pulled into the early endosome through clathrin-mediated endocytosis due to a direct protein-protein interaction of a dileucine motif in T\(\beta\)RII with the clathrin adaptor protein AP-2 [82]. The early endosome, is enriched in an anchoring protein called SARA (Smad Anchor for Receptor Activation), which functions to spatially facilitate the phosphorylation of R-Smads by activated TGF\(\beta\) receptors [87]. Like many other early endosome proteins, SARA contains a FYVE domain, which binds to PtdIns3P, a membrane lipid enriched in early endosomes [7, 87]. SARA binds the R-Smads (Smad2 and Smad3) \textit{via} their MH2 domain, and the carboxy terminal domain of SARA binds to the activated TGF\(\beta\) receptor complex, effectively bridging the receptor and R-Smads [87]. SARA preferentially binds non-phosphorylated forms of Smad2, and it is thought that the activated receptor complex, which is formed at the plasma
membrane, is captured by SARA in the early endosome, and then presents the bound R-Smad to the receptor for phosphorylation [7]. Once Smad2 is phosphorylated, it dissociates from SARA, and then binds Smad4, leading to nuclear translocation and the initiation of transcription [87, 88]. This idea that TGFβ receptors access R-Smads at the early endosome greatly implicates trafficking events in controlling the intensity and duration of Smad phosphorylation. Indeed, maximal signal transduction is dependent on TGFβ receptors reaching the early endosome [89]. More specifically, in this study the authors show that although TGFβ receptors can still phosphorylate Smad2 when clathrin-mediated endocytosis is inhibited, endocytosis was required for Smad2 to fully translocate to the nucleus and mediate transcription [89]. Furthermore, over expression of SARA mutants that lack the FYVE domain mislocalizes Smad2 and inhibits Smad2 phosphorylation and gene transcription [87]. These interesting finding suggests that there is an important spatial regulation of the R-Smad activation and translocation process. Furthermore, these reports identify an important regulatory role for trafficking and the spatial organization of TGFβ receptors and Smads in executing TGFβ dependent transcription. Indeed ongoing work seeks to uncover the pathways, and proteins that control these regulatory endocytic processes and ultimately the TGFβ signal.

In addition to being internalized by clathrin-mediated endocytosis, TGFβ receptors can undergo membrane-raft mediated endocytosis, which targets them to a caveolin positive vesicle [67, 68]. The cytoplasmic tail of TβRI contains a caveolin-binding motif, which facilitates its interaction with the scaffolding domain of caveolin-1, and this interaction ultimately targets receptors to caveolae [85]. In contrast to the early endosome, the cav-1 positive endosome facilitates the interaction of Smad7 instead of Smad2 with the activated receptors [68]. As mentioned, Smad7 is an inhibitory Smad, and has an antagonistic role in TGFβ signalling. More specifically, it sterically prevents the interaction of Smad2 with TβRI through competitive binding thereby preventing its activation and subsequent binding to Smad4 [90, 91]. Furthermore, it acts as an adaptor protein for the ubiquitin ligase Smurf2 that targets the activated receptor complex for degradation through ubiquitination [92]. Ubiquitin is a small 76 amino acid protein that is covalently added to lysine residues of proteins by ubiquitin ligases, which catalyze this post-translational modification. Poly-ubiquitin chains can target proteins for degradation
by the proteasome and can also regulate trafficking events to the lysosome [93]. Ubiquitin mediated protein degradation controls the turnover and stability of various proteins including many cell surface receptors [93]. Interestingly, ubiquitin itself has seven lysine residues to which further ubiquitin molecules can be attached. Mono-ubiquitination and Lys-63 linked polyubiquitination are known to be important regulators of the localization and/or function of proteins, whereas Lys-48 linked polyubiquitination of proteins is a signal for proteasomal degradation [93, 94]. Ubiquitination is facilitated by a multi-enzyme cascade consisting of E1, E2, and E3 ligases (reviewed in [93]). E1 enzymes activate ubiquitin for conjugation, E2 enzymes are ubiquitin conjugating enzymes, and E3 are ubiquitin protein ligases which transfer the ubiquitin chain to the target lysine residue [93]. Smurf2 is a HECT-domain (Homologous to the E6-AP Carboxyl Terminus) E3 ubiquitin ligase, which like other protein members of this class, can directly target ubiquitin to specific substrates. Smurf2 is normally nuclear, but its binding to Smad7 induces export and stable interaction with the activated TGFβ receptor complex [92]. Smurf2 then ubiquitinates the TGFβ receptor-Smad7 complex which leads to their targeted degradation through proteasomal and lysosomal pathways [92]. Thus, TGFβ receptor internalization via membrane rafts into cav-1 positive compartments seems to be an important negative regulator of TGFβ induced Smad signalling. Indeed, receptor trafficking and ubiquitination acts as a tight control mechanism for TGFβ receptor degradation, and subsequently the availability of active receptor complexes.

It has become clear that the method of receptor internalization influences the magnitude, duration and efficiency of TGFβ signal transduction. More specifically, trafficking of the TGFβ receptors into SARA enriched early endosomes facilitates signalling whereas trafficking into cav-1 positive vesicles targets the receptor complex for degradation through the interactions of Smad7 and Smurf2 (Figure 1.4). Thus, the early endosome acts as a signalling platform for the TGFβ receptors that effectively sequesters the receptor away from cav-1 and raft mediated endocytosis and also promotes the access of the substrate Smad2 through its enriched levels of SARA.
Activated TGFβ receptors internalize via clathrin coated pits into the early endosome to facilitate downstream signalling. In the early endosome, SARA presents Smad2 to the activated receptor complex. TβRI phosphorylates Smad2, which stimulates the dissociation from SARA and the subsequent interaction with Smad4. This Smad complex translocates to the nucleus to activate TGFβ target genes. Alternatively, receptors internalize into caveolin-positive vesicles and interact with the inhibitory Smad7 leading to subsequent Smurf dependent ubiquitination and degradation of the receptors. Thus, the function of these internalization pathways is dictated by the resident proteins associated with each compartment, and altered trafficking and internalization of TGFβ receptors can alter TGFβ signalling outcomes.
1.2.5 Regulation of Smads

As mentioned above, R-Smads are direct targets of ligand bound TGFβ receptors, and are the key regulators of TGFβ transcriptional response. Generally, in quiescent cells, Smad2 localizes primarily in the cytoplasm, I-Smads are primarily nuclear, and Smad4 resides in both the nucleus and cytoplasm [6]. Upon phosphorylation, Smad2 forms a heteromeric complex with Smad4 and this complex accumulates in the nucleus where it regulates transcription. Once TGFβ signalling is terminated, R-Smads are rapidly dephosphorylated by PPM1A in the nucleus and exported back to the cytoplasm [95, 96]. Although, classically Smads accumulate in the nucleus following TβRI mediated phosphorylation, it is also clear that R-Smads undergo dynamic shuttling to and from the nucleus allowing for a mechanism to constantly “sense” activated TGFβ receptors (reviewed in [23, 97, 98]). Subsequently, the subcellular localization of Smads is also controlled by various mechanisms, partly owing to the presence of distinct nuclear localization sequences (NLS) and nuclear export sequences (NES) [97].

Although small molecules can passively diffuse into the nucleus, proteins larger than 40 kDa (such as Smads) must be actively transported across the nuclear membrane through the nuclear pore complex (NPC) (reviewed in [99, 100]). The classical nuclear import pathway across the NPC involves the aid of a complex of two receptor transporter proteins, importin-α and importin-β. Importin-α acts as an adaptor that recognizes and binds the characteristic basic residues (arginine and lysine) in the NLS of target proteins. Importin-β binds importin-α-tethered cargo and the entire complex can enter the nucleus through its interactions with nucleoporins, which are proteins that constitute the nuclear pore complex (reviewed in [101]). Target proteins can also bind directly to importin-β, or directly to nucleoporins to permit their nuclear entry. Similarly, nuclear export is largely dependent on the export transporter CRM1 (chromosome region maintenance 1) also sometimes referred to as exportin-1 [100, 101]. CRM1 binds to the leucine rich NES, and then interacts with nucleoporins to permit transport back into the cytosol [100]. However, cargo can also bind nucleoporins directly to permit their export.

The MH1 domain of all Smads contains a conserved NLS, however, the nuclear import of Smad2 occurs independently of Importin-β [102]. Instead, nuclear shuttling of
Smad2 is dependent on a direct interaction of the MH2 domain of Smad2 with nucleoporin proteins. Smad2 directly interacts with CAN/Nup214, a nucleoporin protein that resides on the cytosolic side of the NPC, which facilitates the entry of Smad2 into the nucleus [102]. On the nuclear side of the NPC, Smad2 can bind the nucleoporin Nup153, which facilitates its export from the nucleus [102]. Interestingly, the residency of Smad2 in the nucleus or the cytosol is dependent on cytosolic or nuclear interacting proteins that can act as retention factors. For example, SARA (an endosomal protein) competes with CAN/Nup214 for binding to hydrophobic patches in the MH2 domain of Smad2 [103]. Because receptor mediated phosphorylation of Smad2 decreases its affinity for SARA [88], this may enhance the ability of Smad2 to bind CAN/Nup214 and thus nuclear transport. Once in the nucleus, Smad2 then interacts with transcriptional co-factors, which retain it in the nucleus. Consistent with this idea, expression of the Smad binding domain of SARA, reduces nuclear accumulation of the Smad MH2 domain [88], whereas Smad2 nuclear accumulation is promoted through the expression of the Smad binding nuclear transcription factor FoxH1 [102, 104]. Phosphorylated forms of Smad2 are retained in the nucleus more efficiently than unphosphorylated forms, indicating that phosphorylation increases the association of transcription co-factors and Smad4 binding [23]. Moreover, although the MH1 domain of R-Smads can bind DNA, this intrinsic DNA binding affinity is relatively low and Smad mediated transcription requires other DNA binding transcription factors in a complex with Smads [98]. Thus, the idea that Smad2 can constantly shuttle to and from the nucleus emerges as a mechanism by which the Smads can constantly monitor the activity status of the TGFβ receptors – where regulatory factors such as SARA retain it in the cytoplasm, and nuclear partners retain it in the nucleus for TGFβ mediated transcription.

Interestingly, the nuclear import of Smad4 follows a more classical nature. Smad4 contains a NLS in its MH1 domain, which unlike Smad2, does interact with importin-α [105]. Smad4 has two NESs, one is located in its MH1 domain and the other in its linker region, and nuclear export is mediated by CRM1 [97, 105-107]. Smad4 accumulates in the nucleus through its association with R-Smads, however, evidence for Smad4 shuttling independent of ligand was provided when it was discovered that mutations in the NLS reduces nuclear entry, whereas mutations of the NES promote
nuclear accumulation [105-107]. Upon ligand stimulation, the Smad4 NES is masked by the interaction of Smad4 with the phosphorylated R-Smad, which allows Smad4 to accumulate in the nucleus [95, 106]. Smad4 dissociates from R-Smads once R-Smads are dephosphorylated, and both are exported from the nucleus to the cytoplasm [95]. If TGFβ receptors are still active, R-Smads are phosphorylated, bind Smad4, and return to the nucleus.

Thus, constant nucleo-cytoplasmic shuttling of Smads is a key feature of TGFβ signalling. The continuous cycles of receptor mediated phosphorylation in the cytoplasm, and rapid nuclear dephosphorylation allows for a constant sensing mechanism that detects the activation status of the receptors, and allows for proper termination in the absence of ligand or receptor termination.

Interestingly, other signalling pathways can also converge on Smad signalling to regulate its activities. As mentioned, Smads consist of an MH1 domain and an MH2 domain separated by a “linker” region. As mentioned above, the MH1 domain is involved in DNA binding, and the MH2 domain is involved in binding other protein partners, such as activated receptors, cytoplasmic retention factors, nucleoporins, and nuclear proteins involved in transcription such as co-factors. The linker region in between the MH1 and MH2 domains is variable among the Smads, and has been shown to have various regulatory functions (reviewed in [108]). Interestingly, several ser/thr kinases have been discovered to phosphorylate the Smad linker region. For example, all three members of the mitogen activated protein kinase pathways (MAPK), including extracellular-signal related kinase (ERK), Jun N-terminal kinase (JNK) and p38 kinase have all been shown to phosphorylate Smads in the linker [108]. Initially, ERK was found to phosphorylate the R-Smads 1, 2 and 3 on multiple residues in the linker region and this effectively reduced the ability of R-Smads to accumulate in the nucleus in response to TGFβ [109, 110]. This became an interesting topic, as a properly timed nuclear exclusion of R-Smads through linker phosphorylation was shown to be quite important for early embryonic developmental processes (reviewed in [111]). More specifically, R-Smad linker phosphorylation by MAPK, and subsequent attenuation of TGFβ-Smad signalling, deprives the ability of the ectoderm to become mesoderm, and was also shown to be important to promote the neural differentiation and dorsalization of the mesoderm in
Xenopus frog embryos [112, 113]. Smad linker phosphorylation has also been shown to play a role in oncogenic progression. Many cancer cells are often transformed by oncogenic Ras, which activates mitogenic signalling by ERK, and thus enhances proliferation. Normally, TGFβ signalling can override the proliferative effects of mitogenic Ras signalling in epithelial cells – however, it was discovered that oncogenic Ras signalling to ERK can antagonize TGFβ tumour suppressive effects through Smad linker phosphorylation and nuclear exclusion [110]. However, although these initial reports describe Smad linker phosphorylation by ERK leading to nuclear exclusion, since then multiple studies have also reported that linker phosphorylation (by various kinases) can also enhance TGFβ-dependent transcription [114-117] – seemingly two completely opposite effects. Furthermore, in addition to MAPK members, multiple other kinases such as GSK-3, CDKs, CAMK, and ROCK, have been shown to phosphorylate the Smad linker region regulating various processes in various cell types, indicating that the role of Smad linker phosphorylation is significantly more complicated than initially anticipated [108]. Clearly, linker region phosphorylation does have the ability to modify TGFβ signalling, but the exact mechanisms and outcomes in varying contexts are still unclear and are currently still being investigated.

1.3 Protein Kinase C

1.3.1 The Protein Kinase C Family

Protein kinase C (PKC) consists of a family of ser/thr kinases involved in diverse cellular processes, including cell proliferation, migration, differentiation, apoptosis, neurotransmission, signal transduction and cell polarity (reviewed in [118-124]). The PKC family consists of at least 10 members divided into three subgroups based on their structure and their requirements for activation: the classical PKCs (cPKC), the novel PKCs (nPKC) and the atypical PKCs (aPKC) (Please see Figure 1.5) [118-124].

Classical PKCs (\(\alpha, \beta_1, \beta_2, \gamma\)) require calcium and diacylglycerol (DAG) for activation, novel PKCs (\(\delta, \epsilon, \eta, \theta\)) require DAG, and atypical PKCs (\(\upsilon, \lambda\) and \(\zeta\)) are independent of DAG or calcium [119-123, 125]. All PKCs also utilize phosphatidylerine
as a co-factor for activation [126, 127]. PKC isoforms contain an N-terminal regulatory region, and a C-terminal kinase domain. Within the regulatory region lies an N-terminal psuedosubstrate domain which binds and autoinhibits the kinase domain of the enzyme by mimicking PKC substrate sequences and occupying the substrate binding site [124].

The cPKCs contain two cysteine rich domains. One is termed the C1 domain, which binds DAG and other phospholipids, the other is termed the C2 domain which binds calcium. The nPKCs have a C1 domain but no functional C2 domain, resulting in their calcium insensitivity. These two groups can also be activated by phorbol myristate acetate PMA, which mimics endogenous DAG [128]. The aPKCs lack a C2 domain and their C1 domain is truncated and does not bind DAG (or PMA) – thus rendering them insensitive to calcium or DAG [129, 130]. Instead, aPKCs can be activated by other lipid components such as phosphatidylinositols, phosphatidic acid, arachidonic acid and ceramide or by kinases such as PI3K (phosphatidylinositol 3 kinase) or PDK1 (3’PI-dependent kinase 1) [131-136].

Numerous extracellular signals control the activity status of the various PKC isoforms, which in turn affect the activity of transcription factors, enzymes, cytoskeletal proteins, and cellular receptors. Classically, PKCs are activated through cell surface receptors that trigger intracellular signalling pathways. G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) activate PKC through signalling pathways that produce second messenger molecules such as calcium and DAG [124]. For example, the activation of GPCRs by their extracellular ligands regulates the activity of intracellular G-proteins, some of which can lead to the activation of phospholipase C β (PLCβ). PLCβ triggers the production of inositol 1,4,5 triphosphate (IP₃) and DAG. IP₃ triggers increases in the intracellular levels of calcium, whereas DAG activates both cPKCs and nPKCs. In addition to GPCRs and RTKs, more recently TGFβ ser/thr kinase receptor activity has also been shown to lead to PKC activation [137]. Additionally, other proteins and lipids can influence the regulation of PKCs. For example, arachidonic acid and similar fatty acids are also known to activate PKC [138]. Furthermore, the localization of several PKC isoforms to specific subcellular destinations can be altered through their interactions with RACK proteins (receptors for activated C kinase) which act as escorts to specific areas of the cell [118].
In the basal state, PKCs localize mainly to the cytoplasm in a folded inactive structure. The N-terminal pseudosubstrate domain folds over to bind to the C-terminal catalytic domain of the PKC thereby holding PKC inactive. When intracellular levels of calcium and DAG increase, PKC translocates to the plasma membrane where it can bind these second messengers, which ultimately cause the pseudosubstrate domain to dissociate from the kinase domain. This allows PKC to interact with and phosphorylate its substrates, thereby triggering downstream signalling cascades[124].

Various studies have shown that many PKC isoforms are expressed in virtually all tissue and cell types, and that expression is developmentally regulated and is related to the differentiation status of a tissue (reviewed in [139-141]). Originally it was difficult to attribute specific functions to specific PKC isoforms, due to the similar activator requirements and substrate specificities of the various PKCs in vitro. However, genetic manipulations of specific isoforms have identified PKC isoform specific functions, making it clear that PKCs can execute unique and non-redundant functions within the cell. Consistent with this, PKC isoforms show different subcellular localization, tissue distribution, and binding partner specificity, which all contribute to their differential activations and specified functions [121, 142, 143].
Figure 1.5. Schematic representation of the PKC family

Most of the PKC isoforms have 4 conserved domains (C1-4) that form the diacylglycerol (DAG), calcium, ATP and substrate binding sites. All PKCs also have a pseudosubstrate region (PS) which autoinhibits kinase activity by binding to the substrate binding region of the inactive enzyme. Both novel and atypical PKCs lack amino acids in the C2 region to bind calcium. Atypical PKCs have only 1 cysteine-rich motif, and no detectable DAG binding sites. aPKCs also contain a Phox-Bem 1 (PB1) motifs that are protein-protein interaction domains and will bind to proteins such as Par6.
1.3.2 Protein Kinase C and Membrane Trafficking/Endocytosis

Upon activation, PKCs regulate signalling pathways that control various cellular functions. In particular, PKCs can regulate the endocytosis, membrane trafficking, and desensitization of various receptors. Although the mechanisms are still being uncovered, PKC isoforms may be central in controlling the vesicular trafficking of various transporters, receptors and other plasma membrane proteins (reviewed in [118]). For example, the activation of various GPCRs can lead to the activation of PKCs which initiate downstream signalling events, however, PKCs can also phosphorylate the GPCRs themselves. PKCs can phosphorylate residues within the cytoplasmic loops and C-terminal domains of many GPCRs initiating their desensitization, endocytosis and sometimes degradation [118, 144-147]. Furthermore, it is well known that endocytosis of GPCRs can be further facilitated by the binding of the signal terminator adaptor proteins beta-arrestins to these phosphorylated residues [147, 148]. Internalized receptors can be recycled back to the plasma membrane through either a fast recycling pathway, or they may reside in endosomes for an extended period of time before undergoing a slow recycling, or degradation [148]. Interestingly, roles have emerged for PKCs in both of these pathways thereby implicating PKCs as important mediators of intracellular receptor traffic [146, 149, 150]. The intracellular itinerary of proteins following PKC-activated internalization of receptors can vary. For example, H+/K+ ATPase ion pumps exhibit decreased recycling and activity, whereas β-integrin receptors exhibit increased recycling to the cell surface and enhanced activity [151, 152]. Interestingly, the atypical PKCs (aPKCs) can stimulate the translocation of EGF receptors to late endosomes through the association of aPKC to the late endosome sequestering protein p62 [153]. The atypical PKC isoforms constitute a unique class of PKCs, and interestingly one member (PKCζ) has been implicated in TGFβ processes previously [154, 155].

1.3.3 Atypical Protein Kinase C

The atypical PKCs (aPKC), which consists of PKCγ/λ (λ is the mouse homolog) and PKCζ are a unique set of PKCs that do not require DAG, phosphatidylserine, or
calcium for their activation [156]. This distinctive characteristic is largely due to a unique regulatory domain [125, 157] at the NH2 terminus, which lacks a DAG or calcium binding motif [121, 158]. aPKC plays a critical role in establishing both front-rear and apical-basal cell polarity through its interaction with the polarity complex Par6, Par3, cdc42 and Rac1 [155, 159-161]. PKCζ and PKCλ share a 72% sequence homology at the amino acid level [162], however, they may have some diverging functions. The most striking example of this is that PKCλ knockout is embryonic lethal, whereas PKCζ knockout mice develop normally, although with immunological deficits [130, 158, 163]. It still remains unclear whether the critical role of PKCζ/λ in mouse development is related to the establishment of cell polarity, or to some other critical embryonic function. Interestingly, a third atypical isoform of aPKC, termed PKMζ, was discovered to be expressed in the brain and is known to be important in long term potentiation (LTP) maintenance and memory [164-167]. This brain specific isoform was originally thought to be a cleavage product of full length PKCζ, as PKMζ is essentially the kinase domain of PKCζ and thus lacks an autoinhibitory regulatory domain. However, this constitutively active kinase was later shown to be an alternatively transcribed mRNA in the brain through an internal promoter within the PKCζ gene and not a proteolytic product [168].

Classical mechanisms of activation of aPKCs consist of two main events: release of pseudosubstrate inhibition and phosphorylation of the aPKC kinase domain on Thr-410 by PDK1 and Thr-560 by other unknown kinases or possibly autophosphorylation (reviewed in [130]). As mentioned, unlike the other PKCs, which are released from pseudosubstrate inhibition by DAG, the aPKCs are activated by other lipid components such as phosphatidylinositols, phosphatidic acid, arachidonic acid and ceramide. PIP3 appears to be a major activator of aPKC as its formation contributes to the direct modulation of aPKC pseudosubstrate inhibition and also by activating PDK1 which then phosphorylates the aPKC kinase domain [130]. Furthermore, direct interacting proteins can regulate aPKC function. For example, prostate apoptosis response-4 (Par-4) can interact with regulatory domain of aPKCs and inhibits their activities [169]. Furthermore, the polarity complex protein Par3 (also known as ASIP; aPKC-specific interacting protein) interacts with the kinase domain of aPKCs and inhibits their activity [170]. Another polarity protein, Par6, interacts with aPKC along with Par3 to form the polarity
complex. In this complex, aPKC is held inactive until an active form of Cdc42 binds Par6, thereby leading to activation of aPKC, and phosphorylation of target proteins that drive the establishment of cell polarity [171, 172]. aPKCs have been implicated in functioning in numerous other intracellular signalling pathways including MAPK, NFκB, TGFβ, and Rac1 (reviewed in [130, 163, 172]) and are becoming increasingly important targets in human pathologies.

1.3.4 aPKC in cancer

Early in the 1980s, PKCs were discovered to be the major intracellular receptor of the tumour-promoting phorbol esters, which stimulated a massive effort to define roles of PKCs in oncogenesis [121, 173]. Although variations in PKC localization, activity, phosphorylation, and/or expression have been documented in virtually all tumour types, aPKCs have garnered considerable attention in cancer biology, as the atypical PKCγ is considered to be a human oncogene (reviewed in [158, 174]).

Interestingly, it is possible that aPKC isoforms have cell and tissue specific roles in cancer. For example, increased PKCζ expression has been documented in bladder, hepatocellular, and head and neck carcinomas [175-177]. Similarly, PKCγ overexpression has been reported in breast, ovarian, and liver cancers [178-180] and has been implicated in glioma proliferation and invasion [181-184]. Furthermore, PKCγ is reported to be oncogenic in non-small cell lung cancer (NSCLC) and elevated expression is correlated with poor prognosis in NSCLC patients [157, 158, 160, 185-187]. Between the two isoforms, PKCγ has garnered the most attention with respect to cancer as it has been implicated in the promotion of carcinogenesis in vivo and in vitro and is accepted by some as the first PKC to be a human oncogene [158]. For example, six non-small cell lung cancer cell (NSCLC) lines (A549, H1299, H292, ChaGoK1, Sk-Mes1 and H520) have elevated expression of PKCγ compared to non-transformed lung epithelial cells (HBE4). In NSCLC immunohistochemistry studies show that overexpression of PKCγ is confined to lung tumour cells with little to no expression present in adjacent stroma [160]. The elevation of PKCγ expression in NSCLC patients is predictive of poor
outcome: patients with early stage lung cancer and high PKC\(\alpha\) expression are more than 10 times likely to die from the disease than those with low PKC\(\alpha\) [160]. A similar trend is evident in patients with increased PKC\(\alpha\) DNA copy number and ovarian cancer [178]. Increased PKC\(\alpha\) expression is also correlated with increased cyclin E expression in ovarian cancers, and is implicated with increased proliferation, defects in cell polarity, and overall decreased survival [178]. Expression of a kinase deficient mutant (kdPKC\(\alpha\)) in human A549 lung adenocarcinoma cells results in a loss of anchorage independent growth, but does not affect adherent cell growth, which suggests that PKC\(\alpha\) is essential to driving transformed growth [186]. This effect is also apparent in vivo, as nude mice challenged with NSCLC cells expressing kdPKC\(\alpha\) displayed a reduction in tumourigenicity as compared to mice challenged with wild-type NSCLC tumours [186]. Aurothiomalate (ATM), a gold compound that has been used in the past to treat rheumatoid arthritis, has been found to be a potent inhibitor of the PBI interaction between PKC\(\alpha\) and Par6 [187]; Inhibiting this interaction is thought to inhibit transformed growth of tumours by targeting the PKC\(\alpha\)-Par 6 interaction and at the time of writing, has currently passed phase 1 clinical evaluation for use in non-small cell lung cancer [188].

An earlier study highlighted the pro-apoptotic effects of ATM on aggressive prostate cancer cells by the activation of ERK and p38 MAP kinases [189] indicating that the interaction of aPKC with Par6 may be important for survival of aggressive tumour cells. aPKCs are also considered survival genes, and several studies have highlighted the oncogenic characteristics of PKC\(\alpha\) in NSCLC, ovarian, colon, breast, and pancreatic cancers [157, 160, 185-187, 190, 191], and thus aPKCs may be a viable therapeutic target for various human cancers. Interestingly, aPKC is linked to various TGF\(\beta\) pathways through direct interactions of T\(\beta\)RI binding partners such as the polarity complex protein Par6, and the E3 ubiquitin ligase TRAF6 (Figure 1.6) [5, 192, 193]. Although connected, whether aPKCs alter TGF\(\beta\) receptor functions and their corresponding signalling pathways remains to be elucidated.
The aPKCζ has been shown to associate with the TGFβ receptors through the scaffolding polarity protein Par6. This interaction occurs via PB1 domains contained on both aPKC and Par6. Importantly, PKCζ and Par6 can also bind Smurf1, an ubiquitin ligase that can target proteins for degradation. aPKC can also directly bind TRAF6, a component of the TGFβ-p38MAPK pathway, and p62, another PB1 domain containing scaffold protein. Although, aPKC has been linked to the TGFβ receptors, its role in TGFβ signalling is not understood.
1.4 Par6 and the polarity complex

1.4.1 Cell Polarity

Higher order organisms consist of various specialized cell types that can execute specific functions. For example, neurons have long axons and make hundreds of connections with other cells to create a complex signalling network that can transmit information to peripheral regions of the body. Alternatively, one function of epithelial cells is to regulate and limit the transport of molecules to inner tissues, thereby creating a physiological and mechanical barrier from the outside environment. This asymmetry of cellular function is called ‘cell polarity’ and is created by conserved signalling pathways that create asymmetric distribution of constituents within the cell. Cell polarity can refer to the antero-posterior polarity critical for asymmetric cell division during development, apical-basal polarity of epithelial cells, front-rear polarity seen in polarized migrating cells, or axon formation from naïve neurites seen in neurons (reviewed in [172]). Interestingly, these seemingly very different cell polarity processes are controlled by a set of evolutionarily conserved proteins involving Par6 and aPKC. There are 6 Par genes, Par1 and Par4 are ser/thr kinases, Par2 is a RING-finger protein, Par3 and Par6 are PDZ-domain-containing scaffold proteins, and Par5 is a member of the 14-3-3 family of proteins [172]. These proteins are critical for asymmetrical cell division of the early worm embryo, and regulate fundamental mechanisms regarding cell polarization (reviewed in [194]). Importantly, Par6, aPKC and Par3 form part of the polarity complex in diverse cell types, and this functional unit acts dynamically to regulate cell polarization processes in various contexts in diverse organisms.

1.4.2 Par6 and the polarity complex

Par6 was originally discovered as one of six Par (partitioning-defective) proteins required for the asymmetrical division of the C. elegans embryo [195-197]. Since then, Par6 has been found in metazoans ranging from worms to mammals (reviewed in ref. [194]) and has been established as a mediator in many cellular processes including apical-basal cell polarity, directional cell polarization, cell migration, cell proliferation...
and axonal specification. Par6 is an adaptor molecule for the polarity complex, a highly conserved group of interacting protein partners including aPKC, Par3 and several small GTPases that work in a spatiotemporally co-ordinated effort to generate asymmetry within a cell [161, 170, 171, 198-200]. This scaffolding ability is made possible by various important domains on Par6 including a PB1 domain which binds aPKC, a GTPase binding domain which has a semi-CRIB motif that binds Cdc42 and Rac1, and a PDZ domain which binds Par3 (reviewed in [201, 202]). Many studies have documented the role for the polarity complex as fundamental players in normal cell functions, tissue maintenance and development. For example, the polarity complex controls the formation of tight junctions between epithelial cells, and contributes to apical-basal polarity [170, 203-205]. The plasma membrane of epithelial cells is divided into an apical (facing the extracellular space), and a basolateral domain (the portion of the membrane that forms the base and side surfaces), and these membranes contain different lipid and protein compositions. The membrane domains of epithelial cells are separated by junctional complexes, such as tight junctions, adherens junctions, and desmosomes, which act to physically attach cells to each other and also provide a diffusion barrier to prevent the free movement of solutes through the extracellular space [206]. aPKC and Par6 bind through a PB1-PB1 domain heterodimerization, and this complex becomes activated through the binding of activated Cdc42 or Rac1 to the CRIB domain of Par6 [170, 203, 205]. The active aPKC-Par6 complex then binds Par3 by directly interacting with aPKC kinase domain, and also through a PDZ domain interaction with Par6 [207, 208]. This tertiary complex is linked to junctional complex proteins such as JAM1 (junction adhesion molecule 1) through Par3 and promotes the formation of sub-apical junction formation (reviewed in [209]). Furthermore, active aPKC-Par6 complexes lead to the phosphorylation of LGL (lethal giant larvae), and this aPKC induced phosphorylation cause translocation of LGL to basolateral regions of the cell, and constricts them from being at the apical membrane [204, 210, 211]. This physical restriction of LGL to the basolateral region further potentiates cell polarity by interacting with the basolateral membrane bound proteins discs large 1 (DLG1) and Scribble [212, 213]. Thus, aPKC and Par6 are part of intricate signalling networks that regulate cell polarity through the basis of mutual exclusion and spatial restriction.
Similarly, Par6 and aPKC have been shown to be essential regulators of the polarized migration through the localized activation of Par6-aPKC at the leading edge through binding of the small GTPase Cdc42 [214]. In this model, scratching a monolayer of primary astrocytes leads to a reorganization of the microtubule network and repositioning of the Golgi apparatus, mediated by the integrin stimulated activation of Cdc42 at the leading edge of the migrating cells. Furthermore, the activation of aPKC-Par6 at the leading edge of migrating cells, inhibits the activity of GSK-3β, which promotes the localized association of adenomatous polyposis coli (APC) with the plus ends of microtubules and the formation of DLG1 positive puncta in the plasma membrane [199, 215]. DLG1 interacts with APC directly, and this interaction is required for the microtubule dynamics that regulate cytoskeleton polarization [199]. Thus, the activation of the aPKC-Par6 complex at the leading edge by Cdc42, drives the microtubule dynamics associated with elongation at the leading edge, which is crucial for cell polarization and directional migration [198, 199, 214, 216, 217].

Interestingly, some of these polarity proteins may also play a role in membrane trafficking and endocytosis. Recently, a genome wide screen deciphered that several polarity proteins are critical in regulating membrane trafficking [218, 219]. These include aPKC, Par6, Par3, and the small GTPase, cdc42 [218, 219]. Knockdown of these proteins using small interfering RNA (siRNA) effected changes in clathrin dependent endocytosis, and endosomal recycling [218, 219]. Interestingly, these polarity proteins, once thought to be strictly involved in controlling cell polarity and migration, may also be important players in regulating endocytic traffic.

Although polarity proteins regulate normal cell and tissue polarity, they are often deregulated in tumour cells (reviewed in ref. [220]) indicating that loss in tissue architecture and oncogenesis may go hand in hand. The loss in organization associated with oncogenic transformation is likely to involve changes in the expression, localization and activation patterns of key polarity proteins such as Par6 and aPKC [221]. Indeed Par6 has recently been reported to be overexpressed in breast cancer and enhances the progression of breast cancer through disruption of cellular organization and enhancing proliferation [222, 223] and roles for aPKC in oncogenesis are emerging as described above.
1.4.3 The TGFβ-Par6 axis

The alterations in the normal function of polarity proteins such as Par6 and aPKC may contribute to tumour progression. It is likely that the switch from controlling normal cell polarity to the disruption of tissue architecture may also depend on extrinsic cues in a context dependent manner. One such cue was discovered when a novel screen identified Par6 as a binding partner of the type I receptor of the TGFβ pathway [224]. This interaction proved to be important, as TGFβ activation induced the phosphorylation of Par6 by TβRII on a conserved serine residue (S345) resulting in altered cytoskeletal plasticity and EMT (Figure 1.7) [155]. This phosphorylation stimulated the recruitment of the ubiquitin ligase Smurf1 to target RhoA for degradation at tight junctions. The localized degradation of RhoA was shown to be required for the dissolution of junctional complexes, rearrangement of the actin cytoskeleton, and EMT (Figure 1.7) [155]. Importantly, this pathway is parallel but independent of the classical TGFβ-Smad pathway known to regulate the genetic programme associated with EMT. Furthermore, phosphorylation of Par6 was found to be important in the invasion and metastatic progression of breast cancer cells and consequently has also been correlated with reduced survival in breast cancer patients [223]. Thus, the phosphorylation of Par6 is important in EMT, tumour cell invasion, tissue disruption, and ultimately tumour progression – and until recently, this phosphorylation has only been documented to occur through the TGFβ receptors. Uncovering the details around the TGFβ-Par6 axis with respect to normal cell polarization, and tissue disrupting processes such as EMT is an ongoing area of study.
Figure 1.7. TGFβ receptors phosphorylate Par6 to trigger EMT

TGFβ receptors participate in accessory non-Smad pathways to regulate responses. One such non-Smad pathway is the TGFβ-Par6 axis. The polarity proteins Par6 and PKCζ form a complex with the TGFβ type I receptor at junctional complexes of polarized epithelial cells. Upon TGFβ stimulation, the TGFβ type II receptor is recruited to the complex and phosphorylates Par6 on S345. This induces the recruitment of Smurf1 which ubiquitinates RhoA, a small GTPase critically involved in regulating the actin cytoskeleton, and targets it for degradation. This results in actin cytoskeleton remodeling, dissolution of tight junctions, and the onset of epithelial to mesenchymal transition (EMT) and tumour progression [155, 223].
1.5 The Epithelial to Mesenchymal Transition (EMT)

Normal epithelial cells have several defining characteristics. Firstly, they form organized layers of cells that are closely adjoined and held together by special junctional complexes such as tight junctions, adherens junctions, and desmosomes [225, 226]. Also, epithelial cells have apical-basolateral polarity, are attached to a basement membrane, and maintain complete cell-cell adhesion with their neighbours. Under normal conditions these cells do not detach and move away from the epithelial layer [225, 226]. Mesenchymal cells on the other hand, do not have apical-basal organization, have fibroblast morphology and are highly motile and migratory [225, 226]. Epithelial cells under certain environmental pressures can undergo a shift into a mesenchymal phenotype: a process known as epithelial to mesenchymal transition (EMT) and involves dissolution of junctional complexes, reorganization of the actin cytoskeleton, loss in polarity, and independent motile behavior (Figure 1.8) [155, 225]. EMT is characterized by a shift in cells from an epithelial phenotype expressing epithelial markers such as E-cadherin, Occludins, and Desmosplakin to cells that are more motile expressing fibroblastic markers such as N-cadherin and Fibronectin [225, 226]. EMT, although a critically important process in development [159, 225, 227], marks a major pathological event in cancer biology whereby tumour cells attain an invasive and migratory phenotype and is a prelude to advanced metastatic disease [228, 229]. TGFβ signalling emerged as an important regulator of EMT through its promotion of invasion and metastasis [9, 12, 57] and understanding the mechanisms by which TGFβ causes highly polarized and adjoined epithelial cells to transform into randomly migrating cells is critical to our understanding of tumour development.

1.5.1 Types of EMT

EMT is an evolutionarily conserved mechanism by which polarized epithelial cells, which are normally attached to a basement membrane, acquire a mesenchymal phenotype. This occurs through various biochemical changes leading to a cell phenotype that is characterized by enhanced invasive potential, increased resistance to apoptosis,
and increased deposition of extracellular matrix. The resulting transition yields cells that can degrade the basement membrane and escape the original epithelial layer from which they originated.

Interestingly, EMT has been observed in three distinct biological settings. More specifically, EMT is observed during development, during wound healing, and during tumour progression. Although the exact interplay of cellular signals that dictate the EMTs in these various functional scenarios is not yet clear, it has been proposed that EMTs should be subcategorized into these three distinct types (reviewed in [230]).

Type 1 EMT is the differentiation processes observed during development. For example, EMT is critical for the generation of the embryonic three layered body plan consisting of the endoderm, mesoderm, and ectoderm, which arise through gastrulation [225]. Thus, type 1 EMT is associated with implantation and embryonic gastrulation to generate the mesoderm, endoderm, and mobile neural crest cells. Furthermore, the primitive epithelium during development, and more specifically the epiblast, undergoes an EMT to become the primitive mesenchyme [230]. Importantly, type 1 EMTs neither cause systemic spread via the circulation, nor do they cause fibrosis. Principally, the purpose of type 1 EMT is to generate mesenchymal cells important during development.

While EMT is critical for body patterning and organogenesis during development, the process can also be recapitulated in the adult. A second type of EMT is involved in tissue regeneration, wound healing, and organ fibrosis; this type has been termed type 2 EMT. Type 2 EMTs are part of a repair-associated event that would normally create fibroblasts in order to reconstruct a damaged tissue after trauma or inflammatory injury [230]. Under normal conditions, this EMT is associated with inflammation and should desist once the inflammation has attenuated, as is the case for wound healing. However, persistent inflammation without attenuation can lead to a persistent EMT response leading to tissue fibrosis and eventual organ destruction through excessive ECM deposition and scarring [230]. Essentially, fibrosis can be considered the result of aberrant EMT and wound healing due to persistent inflammation, and this process is known to be involved in the fibrotic disorders of the kidney, liver, and lung [230].

The third type, type 3 EMT, centers on the transition of neoplastic cells. In order for tumour cells to metastasize, they must detach from the primary tumour, invade into
the surrounding tissue or basement membrane, intravasate into the lymphatic or blood system, and extravasate at a distant site [231, 232]. During tumour progression, the ability of stationary epithelial cells to gain mesenchymal phenotype is essential to the metastatic process. Thus, the EMT program involved in the acquisition of invasiveness and metastatic potential of a growing primary tumour constitute type 3 EMTs. Interestingly, type 3 EMTs can occur in cancer cells to a different extent, with some cells retaining certain epithelial traits, while shedding others, whereas some cells can become fully mesenchymal [230]. This gradient in EMT potential is likely related to the biochemical and epigenetic heterogeneity of tumour cells, and although shares some common features with developmental EMT programmes, type 3 EMTs are different than the rigid execution of EMT seen during development.

1.5.2 TGFβ in EMT

Importantly, EMT is stimulated by extracellular activators that lead to complex genetic and intracellular signalling programmes that regulate this process. TGFβ signalling is a well-known activator of EMT and metastasis [233]. TGFβ was first shown to induce EMT in 1994, where the authors show that the stimulation of mammary epithelial cells with TGFβ induced a mesenchymal phenotype through a signal transmitted by the type I TGFβ receptor [234]. Since then, the process of EMT has been studied extensively in vitro, and TGFβ can stimulate the progressive loss of epithelial markers such as E-cadherin, ZO-1, Occludins, and cytokeratin, and the subsequent gain of mesenchymal markers such as N-cadherin, fibronectin, vimentin, and rearrangement of the cell cytoskeleton through various pathways [3, 4, 225, 230, 232, 233, 235]. Although multiple TGFβ pathways can regulate EMT, the downregulation the major adhesion molecule of epithelial cells, E-cadherin, is central to the EMT process. E-cadherin is a transmembrane cellular adhesion receptor, and constitutes the main type of adhesion system in epithelial cells [232]. E-cadherin based junctional complexes stabilize the multicellular architecture of the epithelium, providing a physical link between adjacent cells to maintain the structural integrity and polarized phenotype of epithelia [225, 232].
TGFβ signalling can potently induce the downregulation of E-cadherin through transcriptional repression, but also by destabilizing cellular polarity. More specifically, the TGFβ Smad dependent transcriptional programme induces the expression of the transcription factors SNAI1 (snail) and SNAI2 (slug), which transcriptionally repress the expression of E-cadherin [236-238]. Importantly, TGFβ stimulation can also lead to the destruction of E-cadherin based junctions through the phosphorylation of Par6 (the TGFβ-par6 pathway) as described above [155]. Thus, both Smad dependent, and Smad independent pathways are involved in the stimulation of EMT.

TGFβ is a critical player in EMT during development, and TGFβ isoforms regulate EMTs in the atrioventricular canal of the heart, and are important for the fusion of the palate [225, 233]. Furthermore, upon pathological examination of human cancers, stromal TGFβ is often found at the invasion front, whose cells are characteristic of those that have undergone EMT [233]. Interestingly, TGFβ has also been discovered to promote cancer cells to acquire a stem cell-like phenotype, through the expression of stem cell markers and the acquisition of self-renewal [233, 239-241]. Importantly, TGFβ induced EMT can stimulate metastasis, but also may be important in generating cancer cells with the ability to self-renew (also referred to as cancer stem cells), thereby creating a tumour promoting phenotype [233]. Thus, characterizing the intricate mechanisms and interplay with other pathways by which TGFβ regulates EMT is an important area of research.
Epithelial to mesenchymal transition (EMT) is an important process during development, but marks an important event during tumour progression where cells are becoming more invasive and metastatic. The essential components of EMT include the disruption of cell-cell junctional complexes, (e.g. tight junctions [TJs], adherens junctions [AJs], and desmosomes), the loss of apical basal polarity, and a restructuring of the actin cytoskeleton. Importantly, EMT also involves a shift in epithelial cell expression markers such as E-cadherin, Occludins, and cytokeratins to cells that express mesenchymal markers such as N-cadherin and Fibronectin. Cells that have undergone EMT show an enhanced migratory capacity, increased invasive potential, and an increased resistance to apoptosis. A reverse process, the mesenchymal to epithelial transition (MET), can also occur, allowing for environmentally regulated cell plasticity.
1.6 Non-canonical TGFβ signalling to MAPK

The activation of the Smad signalling cascade by TGFβ is considered the canonical pathway, however, TGFβ receptors can also activate multiple other intracellular signalling cascades. Other pathways and effectors downstream of the TGFβ receptors have been shown to act independently, synergize, or even antagonize classical Smad signalling. TGFβ is known to activate a variety of signalling networks including, the PI3 kinase-Akt pathway, Wnt pathways, Notch pathways, and MAPK pathways (reviewed in [32, 235, 242]. For this thesis, I will focus on MAPK pathways.

Mitogen-activated protein kinases (MAPKs) are signalling components that transduce extracellular stimuli into a range of intracellular responses. There are 3 principle MAPK pathways: ERK, JNK, and p38 - each of which has a complex but apparent role in the development and progression of cancer [243]. In response to various stimuli (including TGFβ) MAPK members become activated through phosphorylation. This phosphorylation occurs through upstream MAP kinase kinases (MAP2Ks), which are activated by MAP kinase kinase kinases (MAP3Ks) [244]. ERKs are classically involved in mitogenic signalling, and often exhibit high expression and activation patterns in various tumours. Furthermore, they are the downstream targets of oncogenic Ras signalling [244]. The JNK and p38 MAPKs are SAPKs (stress associated protein kinases) that have more complicated roles in cancer as they can both inhibit, and/or stimulate tumour progression, depending on cellular context [243, 244].

MAPK pathways generally have various mechanisms by which they respond to TGFβ induction [242]. MAPK signalling may modify the Smads (e.g. by phosphorylation) thereby mediating the activity of Smads as transcription factors by either stimulating or reducing nuclear translocation [235]. TGFβ receptors can activate non-Smad proteins to initiate signalling cascades that run parallel to Smad signalling to regulate a complex gene response [242]. New lines of research are uncovering networks of complex signalling patterns and crosstalk between multiple pathways that converge to elicit a specific physiological TGFβ response.
1.6.1 TGFβ and MAPK activation

TGFβ can activate all three MAPK pathways (Figure 1.9). TGFβ stimulation can activate ERKs through the type I receptor. Briefly, upon TGFβ stimulation, TβRI recruits and directly phosphorylates ShcA, which then associates with the adaptor protein Grb2 and a nucleotide exchange factor Sos, and this complex activates Ras, which then initiates ERK signalling through Raf and Mek. [245]. This pathway is Smad independent, and it is important to mention that the activation occurs at a much lower level than does stimulation with classical ERK activators such as tyrosine kinase activity [245].

TGFβ also activates the other two MAPK pathways, but by reportedly different mechanisms. Both p38 and JNK activation rely on the type I TGFβ receptor and also a MAP3K, TAK1 (TGFβ associated kinase 1). Briefly, upon TGFβ activation TβRI directly associates with an E3 ubiquitin ligase called TRAF6 [192, 193]. TRAF6 becomes lys-63 poly-ubiquitinated, which promotes the association and activation of TAK1 [192]. This process was shown to be important in p38 and JNK activation, as knockdown of TRAF6 using siRNA (small interfering RNA) abrogated this effect [192]. Interestingly, this activation occurred independent of the kinase activity of TβRI, but the E3 ligase activity of TRAF6 and TGFβ stimulation were required for TRAF6 auto-ubiquitination and TAK1 activation [193]. Importantly, the TGFβ-p38 MAPK pathway described has been reported to be important for p38-stimulated apoptosis. Inhibition of the TGFβ-Traf6-p38 axis using pharmacological inhibition or siRNA knockdown blocks the TGFβ induced apoptosis of various epithelial cells [192, 193, 235, 246, 247]. However, signalling through p38 is complex, as in some instances p38 can also stimulate cell growth and survival indicating possible complex interplay of multiple pathways [243].

Interestingly, aPKCs have been shown to play a role in p38 induced apoptosis, as inhibition or knockdown of aPKC sensitizes glioblastoma cells to chemotherapeutic agents via a p38 dependent mechanism [183]. Interestingly, aPKC is a known direct binding partner of TRAF6 and links it to other signalling pathways (such as NFκB) through binding the late endosome marker and PB1 adaptor protein p62 (Figure 1.6) [153, 248]. Whether atypical PKC plays a role in TGFβ induced p38 MAPK signalling is still not clear.
Figure 1.9. TGFβ can activate MAPK pathways independently of Smads.

Activated TGFβ receptors have been reported to activate all three MAPK pathways in a cell-type and context specific manner. TGFβ receptors can activate ERK MAPK through cascade starting with the direct phosphorylation of Shc. ERK signalling is typically mitogenic in epithelial cells. TβRI can also bind the ubiquitin ligase TRAF6 to stimulate its autoubiquitination, which ultimately triggers a cascade that culminates in the phosphorylation and activation of p38 MAPK and JNK. Both p38 MAPK and JNK are generally pro-apoptotic in epithelial cells.
1.7 Purpose of Study, Hypothesis, Aims

1.7.1 Purpose of Study

TGFβ signalling controls a diverse set of cellular processes including cell polarity, wound healing, differentiation, migration and proliferation [6, 7, 23, 24]. Aberrant TGFβ signalling is a hallmark of many epithelial derived cancers [3, 55], and understanding the mechanisms by which TGFβ signalling is impaired would aid our understanding of tumour progression. Normally, TGFβ acts as a tumour suppressor by controlling the growth of the epithelium, but in many tumours TGFβ undergoes a role switch and becomes a metastatic agent through its induction of the mesenchymal transition and the promotion of invasion [3, 4, 55-58]. This loss of TGFβ growth control, followed by TGFβ induced cancer progression is exhibited in lung cancer [56-58]. Non-small cell lung cancer (NSCLC) is the major form of lung cancer, and accounts for the majority of lung cancer related mortalities [249].

Although, TGFβ should normally play an anti-proliferative role in lung tumours, in NSCLC, the growth inhibitory effects of TGFβ are lost, despite these cells producing functional ligands and receptors [250]. Moreover, TGFβ induces NSCLC cell migration and invasion, which are hallmarks of metastatic tumours [56-58]. Therefore, understanding the mechanisms for TGFβ dysfunction is critical to furthering our understanding of lung cancer progression, and ultimately to developing therapies to circumvent altered TGFβ signalling patterns.

Because TGFβ signal propagation is linked to the membrane trafficking of TGFβ receptors [68, 83, 89, 251, 252], elucidating the factors that contribute to altered membrane trafficking is an important area of research. Furthermore, characterizing the mechanisms that drive TGFβ signalling responses in cancer will require the examination of both Smad and Non-Smad signalling pathways.

1.7.2 Rationale

TGFβ tumour suppression is lost in NSCLC, and TGFβ signalling regulates lung tumour metastasis. The mechanisms by which TGFβ signalling is deregulated to become a tumour promoter are unknown. We believe that the deregulated TGFβ signalling
pathway seen in many NSCLC tumours may be related to altered TGFβ receptor trafficking, as well as alterations to both Smad-dependent and Smad-independent pathways (including Par6 and p38 MAPK). Several lines of evidence suggest that polarity proteins such as aPKC and Par6 have aberrant actions in NSCLC, with aPKC being considered an oncogene [157, 158, 160, 185-187, 253]. Furthermore, polarity proteins have recently been described as important regulators of vesicular trafficking [218]. Finally, aPKC is known effector and binding partner of multiple proteins in TGFβ pathways, including Par6 and TRAF6, although its exact role has not been defined. Thus, aberrant aPKC activity and a crosstalk with TGFβ components may contribute to how cells respond to TGFβ signalling (Figure 1.10).

1.7.3 Hypothesis and Aims

My **Hypothesis** is that aPKC regulates TGFβ receptor signalling pathways by:

1) Altering TGFβ receptor internalization to alter Smad-dependent and Smad-independent signalling
2) Altering Par6 phosphorylation leading to increased migration and EMT of lung cancer cells.

This hypothesis was tested through the following 3 major aims:

**AIM 1:** Examine the role of PKC in TGFβ receptor trafficking and signalling.

**AIM 2:** Elucidate the role of the aPKC in the TGFβ-Par6 pathway.

**AIM 3:** Characterize TGFβ and aPKC knockdown dependent gene changes and examine other non-canonical TGFβ pathways such as p38 MAPK.
1.7.4 Overview of Cell Models

This work primarily used A549s and H1299 lung adenocarcinoma cell lines as these are two readily used, and well established NSCLC cell models. These cell lines have been used for in vitro studies of cell migration [254-256], EMT [57, 257-260], as well as in in vivo metastasis mouse models [261, 262]. Furthermore, both of these cell lines have functional and intact TGFβ signalling pathways [263-265]. In addition to the above mentioned NSCLC cell lines, we also used HEK293T (human embryonic kidney) cells and Rat2 fibroblast cells, both of which are reliably used in our lab [266]. HEK293T cells were used for overexpression and interaction studies, as they are highly transfectable and are a key cell line in helping us dissect the molecular mechanisms surrounding the polarity proteins and TGFβ pathway. Rat2 cells are a highly migratory, mesenchymal, fibroblast cell type, and are used here as proof of concept, as they readily migrate, and respond to TGFβ. HEK293T and Rat2 cells are indispensable tools in helping us understand the molecular mechanisms surrounding TGFβ biology. Finally, we also use Mink lung (Mv1Lu) cells which have been used extensively in the past to dissect the molecular mechanisms underlying the biology and signal transduction of the TGFβ receptors. These cells express TGFβ receptors, their associated Smads, and are especially sensitive to TGFβ induced gene induction and phenotypic response. However, the detection of the endogenous expression of TGFβ receptors using immunofluorescence microscopy has been limited by a lack of good quality receptor antibodies. Therefore, we use Mv1Lu cells stably expressing HA-tagged TGFβ type II receptor (HAT cells). These cells express near-endogenous levels of receptors but the HA tag on TβRII allows us to readily study the trafficking of TGFβ receptors from the cell surface into intracellular vesicles using HA antibodies [68, 267, 268].
Figure 1.10. Exploring a role for aPKC in Smad-dependent and Smad-independent pathways

Activated TGFβ receptors can activate Smads, phosphorylate Par6, and trigger the phosphorylation of p38 MAPK. aPKCs have known roles in endocytic trafficking, and are known binding partners of Par6 and TRAF6 and thus have rational connections to the TGFβ pathway. Whether aPKC plays a role in TGFβ signalling processes is investigated in this thesis.
1.8 References


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Chapter 2

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2 Chapter 2

2.1 Chapter Summary

TGFβ signalling is linked to the membrane trafficking of TGFβ receptors. The Protein Kinase C (PKC) family of ser/thr kinases have been implicated in modulating the endocytic processes of various receptors. The present study investigated whether PKC activity plays a role in the trafficking, and signalling of TGFβ receptors, and further explored which PKC isoforms may be responsible for altered TGFβ signalling patterns. Using immunofluorescence microscopy and ¹²⁵I-TGFβ internalization assays, we show that the pharmacological inhibition of PKC activity alters TGFβ receptor trafficking and delays TGFβ receptor degradation. Consistent with these findings, I demonstrate that PKC inhibition extends TGFβ-dependent Smad2 phosphorylation. Previous studies have shown that PKCζ associates with TGFβ receptors to modulate cell plasticity. I therefore used siRNA directed at the atypical PKC isoforms to investigate if reducing PKC₁ and PKCζ protein levels would delay TGFβ receptor degradation and extend TGFβ signalling. Our findings suggest that atypical PKC isoforms regulate TGFβ signalling by altering cell surface TGFβ receptor trafficking and degradation.
2.2 Introduction

The TGFβ signalling pathway is an essential regulator of many cellular processes including epithelial growth control, apoptosis, and the establishment of developmental fate. Alterations in the TGFβ pathway, such as changes in expression, mutations, or altered signalling patterns, have all been linked to pathological disorders of cell growth, such as fibrosis and cancer [1-5].

Although TGFβ signalling is cell type and context dependent, in most normal epithelium, TGFβ typically controls cell growth and division [1, 6-8]. However, in many pathologies, TGFβ signalling is deregulated and can induce gene changes that are associated with an invasive or fibrotic phenotype. Elucidating the factors that contribute to the alteration of the TGFβ signal can shed light on how TGFβ signalling is deregulated in various disease states. One major factor in the progression of the TGFβ signal involves receptor trafficking and endocytosis [9-13].

The classical TGFβ signalling cascade involves the formation of a cell surface receptor complex that is composed of the TGFβ type I and type II receptors (TβRI and TβRII). TβRII is a constitutively active serine/threonine kinase, and phosphorylates TβRI upon ligand binding. Once activated, TβRI further transduces the signal by phosphorylating Smad2 [14, 15].

Ligand binding to cell surface TGFβ receptors induces their internalization from the cell membrane. The activated receptor complex internalizes one of two ways; Receptor endocytosis via a clathrin-dependent mechanism into the early endosome promotes TGFβ-dependent signal transduction, whereas partitioning into membrane rafts facilitates receptor degradation and signal termination [9].

 Trafficking of TGFβ receptors into the early endosome, results in phosphorylation and activation of a TGFβ effector, Smad2. Phosphorylated Smad2 complexes with Smad4, and this unit translocates to the nucleus to mediate TGFβ gene response. Alternatively, trafficking of receptors into caveolin positive membrane rafts leads to the recruitment of the inhibitory Smad7 and the binding of the E3 ubiquitin ligase Smurf2, which target the receptors for proteasomal degradation [9]. Thus, the membrane trafficking of TGFβ receptors plays an important role in the regulation of the TGFβ pathway and altered receptor trafficking may be linked to aberrant TGFβ signal
propagation. One family of proteins that is becoming increasingly important in endocytic trafficking is Protein Kinase C (PKC).

The PKC family consists of at least 10 members divided into three subgroups based on their structure and their requirements for activation. Conventional PKCs (cPKC) require calcium and diacylglycerol (DAG) for activation, novel PKCs (nPKC) depend only on DAG, and atypical PKCs (aPKC) are independent of DAG or calcium. The PKC serine/threonine kinases are involved in diverse cellular processes and signal transduction pathways that control cell proliferation, migration, differentiation and apoptosis [16-20].

Several lines of evidence suggest that PKC carries important roles in controlling the vesicular pathways of various plasma membrane proteins, transporters and receptors ([16, 21-24] reviewed in [25]). Furthermore changes in PKC expression, localization, or activity can lead to changes in receptor phosphorylation, endocytosis, desensitization, and receptor degradation. For example, PKC has been shown to regulate the endocytosis and desensitization of various G protein coupled receptors (GPCRs) through direct phosphorylation [26]. PKC can also alter other membrane proteins, for example, PKC phosphorylation of the epidermal growth factor receptor (EGFR) regulates its intracellular trafficking, shifting it into recycling endosomes instead of a degradative pathway [27]. Similarly, the trafficking of the Dopamine transporter (DAT) has also been shown to be dependent on PKC activation [28], as DAT seems to be targeted for degradation through a PKC-dependent ubiquitination [29, 30]. Given the increasing evidence emerging that PKC is an important regulator of receptor trafficking, we have explored the role of PKC in regulating TGFβ signalling.

It is unknown whether one or more PKC isoforms plays a role in the regulation of TGFβ receptor trafficking. Interestingly, the atypical PKCs (aPKC) PKC\(\text{t}\) and PKC\(\text{z}\) have recently been given considerable interest with respect to cancer, and others have classified PKC\(\text{t}\) to be an oncogene [31-35]. Notably, PKC\(\text{z}\) has already been shown to be a factor in TGFβ induced epithelial to mesenchymal transitions through its involvement in the recruitment of ubiquitin ligases that drive degradation of the small GTPase RhoA, and ultimately epithelial cell plasticity [36, 37]. Thus, the aPKCs are likely candidates for regulating the endocytic trafficking of TGFβ receptors.
In the present study, we show that PKC kinase activity alters TGFβ receptor trafficking, degradation, and ultimately TGFβ signalling. More specifically, we have shown that the inhibition of aPKC isoforms delays receptor degradation and extends TGFβ induced Smad2 phosphorylation.

2.3 Materials and Methods

2.3.1 Antibodies and Reagents

Commercially available antibodies were purchased from the following vendors: primary monoclonal anti-GFP (Living Colors-JL8), anti-Flag (Sigma F3165), anti-HA (Santa Cruz-Y11-SC-805), anti-β-Actin (Sigma-A2668), anti-PKCI (Santa Cruz-SC11399), anti-PKCζ (Cell Signalling Tech-9372), anti-Phospho-Smad2 (Chemicon-AB3849), anti-Smad2/3 (BD Trans-610842), anti-Smad7 (Santa Cruz SC-7004), anti-Myc (Santa Cruz SC-40), anti-EEA1 (BD Trans Labs-610457) and anti-caveolin-1 (BD Trans Labs-610060) were used as per the manufacturers' suggestions. HRP conjugated secondary goat anti-rabbit (Thermo Scientific-31460), goat anti-mouse (Thermo Scientific-31430) and donkey anti-goat (Santa Cruz SC-2020) were used for western blot analysis. Fluorescently conjugated goat anti-mouse (Jackson ImmunoResearch-715225150) and goat anti-rabbit (Jackson ImmunoResearch-711175152) were used for immunofluorescence studies. Protein G-Sepharose was purchased from GE Healthcare. Gö6976 and GF109203X were purchased from Calbiochem. siRNA to human PKCζ (10620319) and PKCι (10620319) were purchased from Invitrogen. The constructs encoding Flag or HA-tagged TGFβ receptors, Myc-Smurf2, Smad7-HA and PKCζ-HA were used as previously described [36, 38]. The GFP-tagged PKCι was a kind gift from Drs. A. Babwah (The Children's Health Research Institute, London ON, Canada) and S. Ferguson (Robarts Research institute, London ON, Canada) [39]. The HA-tagged Ubiquitin construct was a kind gift from Dr. L. Dagnino (University of Western Ontario, London, ON, Canada) [40].
2.3.2 Cell Culture

A549 non-small cell lung cancer (NSCLC), Rat2 fibroblast and HEK293T cell lines were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum. Mink Lung cells stably transected with HA-tagged TβRII (HAT) cells were maintained in Minimal Essential Medium supplemented with 1% Non-essential amino acids and 10% fetal bovine serum, and 0.3% hygromycin. Cells were kept in a humidified tissue culture incubator at 37°C in 5% CO2. Calcium phosphate transfections were carried on cells at approximately 40% confluency, followed by a change in media approximately 24 hours from time of transfection. Cells were serum starved overnight in 0.2% FBS media prior to treatment with 250 pM TGFβ. siRNA transfections were conducted using Lipofectamine RNAi max according to the manufacturer’s protocol. For ubiquitination studies, transfected cells were pretreated with vehicle or GFX for 1 hour followed by the addition of the proteasome inhibitor MG132 (2.5 μM) for 6 hours prior to lysis.

2.3.3 Protein Concentration

Protein concentrations were determined using the Lowry method (Fisher).

2.3.4 Immunoblotting and Immunoprecipitation

Prior to cell lysis, cells were rinsed with phosphate buffered saline (PBS). Cells were lysed in TNTE (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1mM phenylmethylsulfonyl fluoride, and mixture of protease inhibitors [pepstatin, PMSF, NaF and NaPPi]) and centrifuged at 14,000 rpm at 4 °C for 10 min. Aliquots of supernatants were collected for analysis of total protein concentration. For immunoprecipitation, cell lysates were incubated with the indicated primary antibody, followed by incubation with protein-G-sepharose. The precipitates were washed three times with lysis buffer, eluted with Laemmli sample buffer. Proteins were resolved using SDS-PAGE. Proteins were transferred to nitrocellulose followed by blocking in 5% skim
milk, and incubation with primary antibody overnight at 4°C. Following incubation with HRP conjugated secondary antibody, proteins were visualized using West Dura Super Signal ECL (Fisher) and imaged on a VersaDoc Imaging system (BioRad).

2.3.5 Immunofluorescence Microscopy

Receptor internalization experiments were carried out using Mv1Lu cells stably expressing extracellularly HA-tagged TβRII (HAT cells). HAT cells were incubated with anti-HA Fab fragments followed by anti-rabbit-cy3 Fab fragments in the presence of DMSO, Gö6976, or GF 109203X at 4°C. The cells were then incubated at 37°C for 1 hr to allow receptor internalization, fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and incubated with rabbit anti-EEA1 and mouse anti-Cav1 antibodies at 4°C overnight. Following incubation with the appropriate Cy-conjugated secondary antibodies, the early endosome and membrane rafts were visualized by immunofluorescence microscopy using an inverted IX81 Microscope (Olympus, Canada). Co-localization of receptors with EEA1 or Cav-1 were quantitated over 3 experiments using computer software designed and programmed at the Samuel Lunenfeld Research Institute (SLRI) (Toronto, Ontario) and represented as the mean ±SD. At least 30 cells per experiment per treatment were quantified.

2.3.6 Affinity Labeling

Cells were pre-incubated in control media (containing DMSO) or media containing 10 mM Gö6976, or GF 109203X for 1 hour at 37°C, placed on ice and treated with 250 pM ¹²⁵I TGFβ in KRH plus 0.5% bovine serum albumin at 4°C for 2h. Following cross-linking with disuccinimidyl suberate, cells were lysed (time 0) or incubated at 37°C for 2, 4, or 8 hours prior to lysis. Receptors were visualized by SDS-PAGE and quantified using phospho-imager analysis (Amersham Biosciences).
2.3.7 Phospho-Smad Signalling Assays and TGFβ Treatment

Cells were pre-incubated in serum deprived media (0.2% FBS) containing DMSO, Gö6976, or GF 109203X for 1 hr. They were then treated with 250 pM TGFβ for 30 minutes, washed and further incubated in the presence or absence of the PKC inhibitors in serum deprived media for an additional 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE, and immunoblotted for Phospho-Smad2 and total Smad2 levels.

For the 24 hr TGFβ chase experiment, siRNA treated A549 cells were pre-treated with DMSO (control) or SB431542 (10 mM) for 0.5 hours prior to TGFβ treatment for 1 hour. Cells were then washed with PBS 3 times, followed by incubation in low serum media containing DMSO or SB431542 for an additional 24 hours before lysis. Lysates were processed for SDS-PAGE and immunoblotted as described above.

2.3.8 Statistical Analyses

One-way or Two-way ANOVA analyses followed by post-hoc Tukey’s Test were used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism® Software and p values of <0.05 were considered statistically significant.

2.4 Results

2.4.1 Inhibition of PKC alters TGFβ receptor trafficking

TGFβ signal transduction is highly regulated through receptor endocytosis and membrane trafficking. To determine whether PKC had an effect on the internalization of the TGFβ receptors, we pharmacologically inhibited PKC activity and assessed TGFβ receptor endocytosis using Mv1Lu cells stably expressing extracellularly HA-tagged TβRII receptors (HAT cells). Stable expression of HA tagged TβRII in this cell line allows for sensitive detection of the movement of TGFβ receptors into intracellular
vesicles using immunofluorescence microscopy [9, 41]. The cells were cultured in control media, or media containing a classical PKC inhibitor (Gö6976), or a pan PKC kinase inhibitor, GF109203X (Figure 2.1). Cell surface TGFβ receptors were labelled at 4°C and after incubation at 37°C for 1 hour, the cells were fixed, permeabilized and counterstained with markers for the early endosome (using anti-EEA1) or membrane rafts (anti-Cav-1) (Figure 2.1).

In untreated cells we observed that after 1 hr of internalization, TGFβ receptors gained access to the interior of the cell. Approximately half of the receptors co-localized with either the EEA1-positive or Cav-1-positive compartments in equal proportion (Figure 2.1A, quantified in 2.1D). The cPKC inhibitor, Gö6976, did not alter the ratio of receptors in the EEA1-positive or Cav-1-positive compartments (Figure 2.1B). We did however notice a general accumulation of receptors and Cav-1 positive vesicles at the peri-plasma region of the cell (Figure 2.1B). Interestingly, the pan PKC inhibitor, GF 109203X, did not perturb the position of the Cav-1 compartment but increased TGFβ receptor co-localization with the EEA1 compartment (Figure 2.1C and 2.1D).

These results suggest that the inhibition of different PKC isoforms will alter receptor trafficking. Indeed, pan PKC inhibition shifted the ratio of receptors into the EEA1-positive signalling endosomes whereas targeting classical PKC isoforms did not (Figure 2.1D). From these results we predicted that the rate of receptor degradation, and possibly signal transduction, would be affected. We first assessed whether the alteration in receptor internalization would lead to changes in the rate of TGFβ receptor degradation.
Figure 2.1
Figure 2.1. PKC inhibition alters TGFβ receptor trafficking

Mv1Lu cells stably expressing extracellularly HA-tagged TβRII were incubated with anti-HA Fab fragments followed by anti-rabbit-cy3 Fab fragments in the presence of DMSO (Control; A), 10 μM Gö6976 (B), or 10 μM GF109203X (C). The cells were then incubated at 37°C for 1 hr (to allow receptor internalization), fixed, permeabilized and incubated with mouse anti-EEA1 and rabbit anti-Cav1 antibodies. Following incubation with the appropriate secondary antibodies, the receptors (red), the early endosomal compartment (EEA1; blue) and membrane rafts (Cav-1; green) were visualized by immunofluorescence microscopy. Co-localization of receptors with EEA1 or Cav1 is indicated with arrowheads or arrows, respectively. Note that Gö6976 induces an accumulation of receptors and Cav1-positive structures at the peri-plasma membrane (grey arrowheads; B). Bar = 10 μm.

(D) Receptors co-localizing with EEA1 or Cav1 positive vesicles were quantitated from three separate experiments and represented as the mean +/- SD. (n=3).
2.4.2 Inhibition of PKC activity extends TGFβ receptor half-life

To determine whether PKC kinase activity had an effect on TGFβ receptor degradation, we used $^{125}$I-radiolabelled TGFβ to conduct receptor half-life studies. Briefly, serum starved Mv1Lu cells were treated with $^{125}$I-TGFβ at 4°C; a temperature where TGFβ receptors halted at the cell surface. After cross-linking the $^{125}$I-TGFβ to cell surface receptors, the cells were incubated in control media or media containing PKC inhibitors (Gö6976 or GF109203X) at 37°C. Cells lysates were then processed for SDS-PAGE, and receptor levels are analyzed using phospho-imaging (Figure 2.2). We observed that the inhibition of PKC kinase activity with either inhibitor decreased the rate of TGFβ receptor degradation (Figure 2.2A). The receptor half-life in untreated cells was 2.4 ± 0.3 hours and extended to 4.4 ± 0.8 in Gö6976-treated and 6.6 ± 1.7 in GFX-treated cells (Figure 2.2B). Interestingly, receptor degradation was reduced in the presence of either classical or pan PKC inhibitors.

After observing that PKC inhibition could alter receptor degradation and receptor trafficking, we next addressed if PKC inhibition would affect TGFβ dependent Smad2 phosphorylation.
Figure 2.2
Figure 2.2. PKC inhibition extends TGFβ receptor half-life

(A) Mv1Lu cells incubated in the presence of DMSO (control), 10 μM Gö6976 or 10 μM GF109203X were affinity labelled with $^{125}$I-TGFβ, cross-linked and incubated at 37°C for 0, 2, 4, or 8 hours. Cells were then lysed, and subjected to SDS-PAGE followed by phospho-imaging. The relative mobilities of $^{125}$I-TGFβ-bound TβRII (~90 kDa) and $^{125}$I-TGFβ-bound TβRI (~65 kDa) are indicated.

(B) Three separate experiments as described in Panel A were carried out and the amount of the receptors was quantitated using QuantityOne software and plotted as a percentage of receptors at time 0. The mean +/- SD is shown. (n=3).
2.4.3 TGFβ dependent Smad phosphorylation is extended with PKC inhibition

Smad2 phosphorylation is a key component in TGFβ signalling and is the central mediator of TGFβ-dependent transcription. To assess whether PKC could alter TGFβ dependent Smad signalling, we assessed Smad2 phosphorylation levels over a 4-hour time course (Figure 2.3). Briefly, HAT, Rat2 fibroblast or A549 human lung adenocarcinoma cells were treated for 1 hr with DMSO (control), Gö6976 or GF109203X, prior to TGFβ stimulation for 0.5 hr. After washing out the TGFβ, cells were incubated in the respective serum-deprived media in the presence or absence of inhibitors for an additional 1 or 4 hr before they were lysed (Figure 2.3A). Activated TGFβ receptors will continuously signal until degraded once internalized, thus washout of TGFβ allows for analysis of the duration of TGFβ signalling of internalized receptors. Quantitation showed that HAT cells that were incubated in media containing PKC inhibitors had prolonged levels of Smad2 phosphorylation compared to vehicle-treated cells (Fig 2.3B). These results were not cell line or cell type specific as Rat2 fibroblasts and A549 human NSCLC cells also showed increased and prolonged Smad2 phosphorylation in the presence of PKC inhibitors (Figure 2.3C and 2.3D). We next sought to further determine which class of PKCs was responsible for this extension.
Figure 2.3
Figure 2.3. PKC inhibition extends TGFβ induced Smad2 phosphorylation

(A) Schematic of the experimental procedure to assess phosphorylated Smad2 levels in PKC inhibited cells. HAT (B) Rat2 (C) or A549 (D) cells were pre-incubated in media containing 10 μM Gö6976, 10 μM GF109203X, or DMSO (vehicle). They were then treated with 250 pM TGFβ for 0 or 30 minutes, washed and further incubated in the presence or absence of the PKC inhibitors for an additional 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with anti (α)-phospho-specific Smad2 or Smad2/3 antibodies as indicated. Accompanying densitometrical analyses of average P-Smad2 levels from at least 3 independent replicate experiments is presented graphically for each representative immunoblot. The mean +/- SEM is shown, Two-way ANOVA, *p <0.05.
2.4.4 Targeting aPKC kinases extends Smad2 phosphorylation

Although we observed that both types of PKC inhibitors could extend Smad2 phosphorylation, the signalling pattern was much more prominent in cells inhibited with the broad PKC inhibitor GF109203X compared to the classical PKC inhibitor. This suggested that classical PKC isoforms may play a more subtle role in TGFβ signalling compared to either the novel or aPKC isoforms. To test whether aPKC isoforms were the likely candidates in dampening TGFβ signalling, we carried out similar signalling assays as described above in A549 cells (Figure 2.4). For this assay we incubated cells with two different concentrations of GF109203X: 1 μM, which only effectively inhibits the classical and novel PKC isoforms, and 10 μM, which inhibits all classes of PKCs. Our results demonstrated that signalling was more prominently extended in cells inhibited with 10 μM GF109203X, suggesting that aPKC kinase activity was important in regulating TGFβ signalling.

To confirm our pharmacological observations, we next assessed the effects of PKC isoforms on TGFβ-dependent receptor signalling and degradation via overexpression and siRNA studies.
Figure 2.4
Figure 2.4. aPKC inhibition extends TGFβ induced Smad2 phosphorylation

A549 cells were incubated with 1 μM (inhibits classical and novel PKCs) or 10 μM (inhibits all PKCs) GF109203X for 1 hour. They were then treated with 250 pM TGFβ for 0 or 30 minutes, washed and further incubated in the presence or absence of the PKC inhibitor for an additional 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with anti phospho-specific Smad2 or Smad2/3 antibodies as indicated. Accompanying densitometrical analysis of average P-Smad2 levels from 3 independent replicate experiments is presented graphically for each representative immunoblot. The mean +/- SEM is shown, n=3, Two-way ANOVA, *p <0.05. The asterisk (*) beside immunoblots indicates a second band underneath Smad2 in A549 cells that may represent Smad3 in this cell line.
2.4.5 aPKC over-expression reduces TGFβ receptor steady state levels

TGFβ receptor degradation may be a key determinant in the extension of Smad2 signalling. Our data suggested that aPKCs may be primarily responsible for the negative regulation of TGFβ receptors. To test this, we expressed TGFβ receptors and increasing amounts of a representative member of the classical PKC family, PKCα, or increasing amounts of an aPKC, PKCι into HEK 293T cells (Figure 2.5A). Western blot analysis indicated that PKCι decreased the levels of steady state TGFβ receptors. Of note, the multiple bands that represent the core and glycosylated forms of TβRII receptors as well as TβRI were reduced in the presence of increasing levels of PKCι. In contrast, no apparent changes in receptor levels were noted with increasing expression of the classical PKC, PKCα. Quantitation confirmed that aPKC, but not the cPKC, reduced receptor levels (Figure 2.5B).

To test if aPKC kinase activity was necessary for altering receptor levels, we conducted similar experiments with increasing expression levels of wild-type (WT) or kinase-deficient (KR) versions of PKCζ. Our results showed that increasing amounts of PKCζ lead to decreased steady-state levels of TGFβ receptors. In contrast, increasing amounts of the kinase-deficient PKCζ did not alter TβRI or TβRII expression (Figure 2.5C and 2.5D). We next assessed TGFβ receptor ubiquitination levels.
Figure 2.5
Figure 2.5. aPKC overexpression reduces steady state TGFβ receptor levels

(A) HEK 293T cells were transiently transfected with cDNA encoding the indicated proteins. Equal amounts of TβRI-FLAG and TβRII-HA were co-transfected with increasing amounts of either GFP tagged PKCα, or PKCζ. 48 hours post-transfection, cells were lysed and processed for SDS-PAGE and immunoblotting to assess receptor levels. A representative immunoblot from three separate independent trials is shown.

(B) Average densitometrical quantitation of TGFβ receptors was carried out from 3 separate experiments using QuantityOne software and graphed (n=3).

(C) HEK 293T cells were transiently transfected with cDNA encoding the indicated proteins. Equal amounts of TβRI-FLAG and TβRII-HA were co-transfected with increasing amounts of either HA tagged PKCζ-WT or a kinase deficient version, PKCζ-KR. 48 hours post-transfection, cells were lysed and processed for SDS-PAGE and immunoblotting to assess receptor levels. A representative immunoblot from three separate independent trials is shown.

(D) Average densitometrical quantitation of TGFβ receptors was carried out from 3 separate experiments using QuantityOne software and graphed (n=3).
2.4.6 PKC inhibition alters TGFβ receptor ubiquitination levels

TGFβ receptors can be degraded through the conjugation of ubiquitin to activated receptor complexes by the E3 ubiquitin ligase Smurf2 [38]. Therefore, we next addressed whether PKC inhibition could affect receptor ubiquitination levels (Figure 2.6). HEK293T cells expressing receptors, ubiquitin, Smad7 and Smurf2 were incubated in the presence or absence of GF109203X. Following co-expression of receptors with ubiquitin and wild type Smurf2 and Smad7, the steady state levels of the TGFβ receptors, as well as Smurf2 and Smad7 in the total cell lysates was reduced (Figure 2.6, lower panel lane 3). This was paralleled with an increase in high molecular weight ubiquitinated complexes in the receptor immunoprecipitations (Figure 2.6, top panel lane 3). In contrast, receptors that were co-expressed with a catalytically inactive mutant of Smurf2 (Smurf2-CA) were protected. Interestingly, the levels of the higher molecular weight ubiquitinated complexes increased in cells treated with PKC inhibitor (Figure 2.6, top panel lane 5). Taken together these findings suggested that aPKC kinase activity might be altering TGFβ receptor trafficking to enhance degradation, and inhibiting aPKC results in an increase of ubiquitinated receptors. To test this hypothesis, we next used small interfering RNA directed at the aPKCs and examined cell surface TGFβ receptor degradation.
Figure 2.6
Figure 2.6. PKC inhibition reduces clearance of ubiquitinated TGFβ receptor complexes

HEK 293T cells expressing combinations of TβRII, TβRI-Flag, Ubiquitin-HA, Smad7-HA, and the wild-type (WT) or catalytically inactive (CA) E3 ubiquitin ligase, Myc-Smurf2, were lysed, immunoprecipitated using anti-Flag monoclonal antibodies to isolate TGFβ receptors. The immunoprecipitates were then subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. Anti-HA immunoblotting was used to detect ubiquitinated receptors and/or associated proteins (denoted by the asterisk). Total lysates are shown in the bottom panel. Cells were pre-treated with vehicle or GF109203X for 1 hour followed by addition of the proteasome inhibitor MG132 for 6 hours prior to lysis. A representative immunoblot from 3 independent replicate experiments is presented (n=3).
2.4.7 siRNA targeting aPKC reduces TGFβ receptor degradation

We next addressed whether endogenous aPKC, and which isoform, regulates the degradation of endogenous TGFβ receptors. To ensure that we were able to detect each aPKC specifically, we first tested our antibody specificity in HEK293T cells to test for possible cross-reaction of isoforms (Figure 2.7A). By western blotting, we observed that both the PKCι and PKCζ antibodies were specific. We next assessed our siRNA targeting of the two aPKC isoforms was specific and effective. Our results showed that the siRNA constructs are specific for their targets, however, we also noted that there is a compensation effect for PKCζ, when we silence PKCι: In siPKCι knock down cells, there was a small, yet consistent, increase in expression of PKCζ (Figure 2.7B). Having ascertained that the siRNA to the different aPKC isoforms was specific and effective, we assessed their influence on TGFβ receptor degradation (Figure 2.7C). Consistent with our observations using pharmacological inhibitors, we observed that silencing aPKC isoforms with siRNA resulted in a significant reduction in the degradation of cell surface TGFβ receptors after 8 hours (Figure 2.7C and 2.7D).
Figure 2.7
Figure 2.7. aPKC knockdown reduces TGFβ receptor degradation

(A) Lysates from HEK 293T cells expressing GFP tagged PKCα or PKCζ were subjected to SDS-PAGE followed by immunoblotting with antibodies directed at GFP, PKCα, PKCζ and Actin as a loading control.

(B) A549 cells were transfected with siRNA directed at PKCα, PKCζ, both PKCα and PKCζ (PKCα/ζ) or control siRNA. Cells were lysed and subjected to SDS-PAGE and immunoblotting to assay for PKCα or PKCζ protein expression. Note: Both PKCα and PKCζ siRNAs are effective in all 3 conditions.

(C) A549 cells transfected with the indicated siRNA were affinity labelled with 125I-TGFβ, cross-linked and incubated at 37°C for 0, 2, 4 or 8 hours. Cells were then lysed, and subjected to SDS-PAGE followed by phospho-imaging analysis. The relative mobilities of 125I-TGFβ-bound TβRII (~90 kDa) and 125I-TGFβ-bound TβRI (~65 kDa) are indicated.

(D) Three separate experiments as described in Panel C were carried out and the amount of the receptors was quantitated using QuantityOne software and plotted as a percentage of receptors at time 0. The mean +/- SD is shown, n=3, Two-way ANOVA, *p<0.05.
2.4.8 Knockdown of aPKC extends Smad2 phosphorylation

Using PKC kinase inhibitors and PKC overexpression, we observed that aPKC expression increases receptor degradation and aPKC activity also negatively regulates TGFβ induced Smad2 signalling. Furthermore, knockdown of aPKC using siRNA results in reduced TGFβ receptor degradation. Next we used siRNA to test whether knockdown of specific aPKCs could extend TGFβ induced signalling. After transfecting A549 cells with combinations of siRNA directed towards aPKC isoforms, we conducted Smad2 phosphorylation time courses (Figure 2.8). Our results indicate that in cells where both aPKCs were knocked down (siPKCι/ζ), there was an extension of phospho-Smad2 levels compared to cells transfected with control siRNA. This extension was also seen in siPKCζ cells, but less pronounced in siPKCι cells, indicating that silencing both aPKCs had the greatest effect on phospho-Smad2 levels (Figure 2.8). We next tested to see whether aPKC knockdown could cause longer phospho-Smad2 levels.
Figure 2.8. aPKC knockdown extends Smad2 phosphorylation

A549 cells transfected with the indicated siRNA were serum starved and treated with 250 pM TGFβ for 30 minutes, washed, and further incubated for 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with anti phosphospecific Smad2 or Smad2/3 antibodies. Accompanying densitometrical analysis of P-Smad2 levels from 3 independent replicate experiments is presented graphically for each representative immunoblot. The mean +/- SEM is shown, n=3, Two-way ANOVA, *p <0.05.
2.4.9 Smad2 phosphorylation persists 24 hours in aPKC silenced cells

To test if phospho-Smad2 levels could persist for an extended period of time, we stimulated siRNA treated cells with TGFβ for 1 hour, followed by washout and further incubation of cells for 24 hours (Figure 2.9). Interestingly, we found that in knock down cells, phospho-Smad2 was maintained even after cells were washed of exogenously added ligand. Finally, to test whether any extended Phospho-Smad2 levels were specifically due to the activation of TGFβ receptors, we incubated cells with the TβRI kinase inhibitor, SB431542. This inhibitor blocks TβRI from phosphorylating Smad2, and thus should reduce TGFβ induced phospho-Smad2 signalling. As expected, the extension in phospho-Smad2 levels was abrogated when cells were co-treated with SB431542, indicating that any changes we observed in signalling were TGFβ receptor dependent (Figure 2.9).

Taken together, our data suggest that aPKC isoforms regulate TGFβ signal transduction by regulating receptor trafficking and degradation, summarized in Figure 2.10.
Figure 2.9. aPKC knockdown extends TGFβ induced Smad2 phosphorylation even after 24 hours

siRNA transfected A549 cells were serum starved and pre-treated with vehicle (DMSO) or 1 μM of the TβRI kinase inhibitor, SB431542. Cells were then treated with 250 pM TGFβ for 1 hour, washed and further incubated for 24 hours in control or SB431542 media prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with anti phospho-specific Smad2 or Smad2/3 antibodies. Accompanying densitometrical analysis of P-Smad2 levels from 3 independent replicate experiments is presented graphically for each representative immunoblot. The mean +/- SEM is shown, n=3, Two-Way ANOVA, *p <0.05.
Figure 2.10. Regulation of TGFβ receptor trafficking and signalling by atypical protein kinase C

Pharmacological inhibition of all PKC isoforms alters TGFβ receptor trafficking by shifting receptors into the early endosome, reducing receptor degradation, and extending TGFβ-induced Smad2 phosphorylation. siRNA knockdown of atypical PKC isoforms also reduces TGFβ receptor degradation and extends TGFβ induced Smad2 phosphorylation.
2.5 Discussion

The TGFβ signalling pathway controls many biological responses orchestrated through the regulation of various genes and downstream effectors. Altered TGFβ signalling patterns are often a hallmark in epithelial derived cancers. In certain tumours the proteins involved in TGFβ signalling are still intact and functional, but the pathway is deregulated by some other means. This seems to be the case in non-small cell lung cancer (NSCLC) as many of these tumours are unresponsive to the tumour suppressive properties of TGFβ, despite producing functional ligands and receptors [42, 43]. One proposed mechanism is a change in TGFβ receptor signalling patterns due to altered membrane trafficking of the TGFβ receptors upon internalization. PKCs, which are major regulators of intracellular trafficking, may therefore regulate TGFβ signalling. In the present study we propose that atypical PKC isoforms are involved in altering the vesicular fate of TGFβ receptors to ultimately change TGFβ signalling patterns.

We have shown that PKC kinase activity plays a role in the membrane trafficking and degradation of TGFβ receptors, as broad PKC inhibition with GF109203X caused a shift of receptors into the early endosome and a reduction in TGFβ receptor degradation. Interestingly, the classical PKC inhibitor, Gö6976, did not shift receptors into the early endosome, but did however alter the trafficking of Cav-1 positive vesicles and reduced TGFβ receptor degradation. This brings up the possibility that classical PKC isoforms alter general membrane trafficking and we suspect that this would have an effect not only on TGFβ receptor trafficking but other cell membrane proteins as well.

We observed that the overexpression of aPKC isoforms negatively regulate the steady state levels of both the type I and type II TGFβ receptors. This is in contrast to steady receptor levels when a classical PKC (PKCα) or a kinase deficient aPKC are overexpressed.

Furthermore, using siRNA targeted to aPKC isoforms, we observed that knockdown of aPKC expression results in a reduction in TGFβ receptor degradation from the cell surface. These data suggest that aPKC promotes TGFβ receptor degradation upon endocytosis. Interestingly, the PKCζ knockdown and the double PKCι/ζ knockdown seem to produce the greatest effect. However, it is important to note that in siRNA treated cells where PKCι has been silenced, we observed a consistent increase in PKCζ.
expression, which may be responsible for the less prominent effects on Smad2 phosphorylation. We reason that both aPKC isoforms are capable of increasing TGFβ receptor degradation as double knockdown of PKC\(\eta/\zeta\) produces the greatest effect on temporal extension of TGFβ induced P-Smad2 levels.

Interestingly, although we observe reductions in TGFβ receptor cell surface degradation with PKC inhibition, Smurf2 mediated ubiquitination of TGFβ receptors was increased. This may be the result of an accumulation of ubiquitinated complexes due to an increased residence time of receptors in intracellular compartment(s) and is consistent with our observations that PKC inhibition can delay receptor degradation and extend the duration of phosphorylated Smad2 levels.

This study addresses the idea that aPKC can alter TGFβ receptor trafficking and also alter local TGFβ signalling patterns in A549 lung cancer cells after an initial short TGFβ stimulus. It will be interesting to see whether aPKC knockdown can alter phenotypic changes in cells that receive constant TGFβ stimulation; similar to tumour cells growing in a microenvironment where autocrine and paracrine TGFβ overproduction leads to higher and constant levels of TGFβ stimulation.

Aberrant aPKC expression and activity are becoming more apparent in various cancers [32, 34, 44-46]. More specifically, in NSCLC where an increased expression of aPKC is reported, it is plausible that the TGFβ receptor signalling pathway may be altered by an interplay with aPKC. More specifically, our data suggest that enhanced aPKC expression and activity may contribute to increases in TGFβ receptor degradation, alterations in the TGFβ signal, and consequently a loss in some of the characteristic TGFβ tumour suppressive properties. This is supported by the fact that aPKC localize and can be anchored to late endosomes that are targeted for degradation [24]. Furthermore, since we have observed that aPKC kinase activity is important for changes in TGFβ receptor trafficking, degradation, and signalling, there exists the possibility that aPKC directs TGFβ receptor trafficking into specific compartments through the phosphorylation of one or more of the TGFβ receptors, as is the case for the EGFR [27]. This is supported by the finding that PKC\(\zeta\) is a TGFβ receptor interacting partner in a
complex which regulates cytoskeletal changes involved in epithelial to mesenchymal transition [36, 37].

2.6 Footnotes

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2.7 References


Chapter 3

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3 Chapter 3

3.1 Chapter Summary

EMT is controlled by cellular signalling pathways that trigger the loss of cell-cell adhesion and lead to the restructuring of the cell cytoskeleton. TGFβ has been shown to regulate cell plasticity through the phosphorylation of Par6 on a conserved serine residue (S345) by the type II TGFβ receptor. Here we show that aPKC isoforms are an essential component to this signalling pathway in non-small cell lung cancer (NSCLC) cells. We show that the aPKC, PKCγ, interacts with TGFβ receptors through Par6, and that these proteins localize to the leading edge of migrating cells. Furthermore, Par6 phosphorylation on Ser 345 by TGFβ receptors is enhanced in the presence of aPKC. aPKC kinase activity as well as association with Par6 were found to be important for Par6 phosphorylation. In effect, siRNA-targeting aPKC reduces TGFβ-induced RhoA and E-cadherin loss, cell morphology changes, stress fibre production and the migration of NSCLC cells. Interestingly, re-introduction of a phospho-mimetic Par6 (Par6-S345E) into aPKC-silenced cells rescues both RhoA and E-cadherin loss with TGFβ stimulation. In conclusion, our results suggest that aPKCs co-operate with TGFβ receptors to regulate phospho-Par6-dependent EMT and cell migration.
3.2 Introduction

During tumour progression, EMT characterizes an event where the cohesive, apico-basolaterally polarized cells of the epithelium detach from the basement membrane and acquire the ability for independent movement as mesenchymal like cells [1-3]. EMT is characterized by the loss of E-cadherin based adherens junctions allowing for stationary carcinoma cells to escape the physical constraints of cell-cell adhesion leading to invasion of the stromal compartment [3]. The process of EMT involves altering cell genetic programs and inducing morphological changes that foster an invasive and migratory phenotype [1, 2, 4-6]. TGFβ signalling is an important regulator of EMT through its promotion of invasion and metastasis [7-9]. In conjunction to the canonical TGFβ-Smad pathway that has been shown to alter transcriptional responses leading to EMT, a second TGFβ pathway was defined, in which the conserved polarity protein Par6 was shown to be a binding partner and substrate of the TGFβ receptors [10, 11]. Indeed, TGFβ-dependent phosphorylation of Par6 on Serine 345 leads to Smurf1 mediated degradation of RhoA. This in turn leads to significant remodelling of the actin cytoskeleton, and the dissolution of tight and adherens junctions leading to EMT and metastasis [10, 12, 13].

Par6 is an adaptor molecule for the polarity complex [14-18]; a highly conserved group of interacting protein partners, including aPKC, Par3, and several small GTPases that work in concert to control apical-basal cell polarity, directional cell polarization, migration, and cell proliferation [14, 17, 19-24]. Notably, aPKC was shown to be part of the complex that regulates protrusion formation through the TGFβ receptors [10, 25, 26] although its exact role has not been defined.

The aPKCs, which consist of PKCτ and PKCζ, are a unique subset of PKCs that do not require diacylglycerol (DAG), phosphatidylserine, or calcium for their activation [27]. PKCτ has been implicated in carcinogenesis [28-34] and is considered to be the first member of the PKC family to be a human oncogene [29]. Our recent work has shown that PKC activity can regulate the trafficking and degradation of TGFβ receptors as well as the duration of Phoshpho-Smad2 signalling [35].
Here, we report that in addition to TGFβ receptors, aPKCs phosphorylate Par6 on Ser 345, and aPKC expression increases Par6 steady state levels. Furthermore, reduction in aPKC expression or the association of aPKC with Par6 reduces EMT and migration of NSCLC cells.

3.3 Materials and Methods

3.3.1 Antibodies and Reagents

Primary monoclonal anti-GFP (Living Colors-JL8), anti-Flag (Sigma F3165), anti-HA (Santa Cruz-Y11-SC-805), anti-β-Actin (Sigma-A2668), anti-PKCα (Santa Cruz-SC11399)/ (BD Transduction-610175), anti-PKCζ and anti-PPKCζ (Cell Signalling Tech-9372 and -9378), anti-Rac1 (BD Transduction-610650), and anti-E-cadherin (BD Transduction-610182 and Cell Signalling Tech-3195) were used as per the manufacturers' suggestions. Anti-P-Par6 (S345) was a gift from Dr. Jeff Wrana. HRP conjugated secondary goat-anti-rabbit (Thermo Scientific -31460) and goat-anti-mouse (Thermo Scientific -31430) were used for immunoblot analysis. Fluorescently conjugated goat α-mouse (Jackson ImmunoResearch-715225150), goat α-rabbit (Jackson ImmunoResearch-711175152) and Cy3 conjugated Streptavidin (Jackson ImmunoResearch-016160084) were used for immunofluorescence studies. A555 conjugated Phalloidin (Invitrogen-A34055) was used for F-actin staining.. Human siRNA constructs were purchased from Invitrogen (Stealth) (siPKCζ, siPKCα and siControl catalogue numbers: (10620319-HSS183348, 10620319-HSS183318, 12935112) respectively. The constructs encoding Flag or HA-tagged TGFβ receptors, Flag-Smurf1, Flag-Par6 (WT and S345), PKCζ-HA were used as previously described [10, 36]. The GFP-tagged PKCα was a kind gift from Drs. A. Babwah (The Children's Health Research Institute, London ON, Canada) and S. Ferguson (Robarts Research institute, London ON, Canada) [37]. Transwell migration assays were conducted using Costar transwell permeable support inserts with a pore size of 8 μm (Costar-3422).
3.3.2 Cell Culture and Transfections

Rat2 fibroblast, and HEK293T cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. A549 and H1299 NSCLC cell lines were maintained in F12K and RPMI-1640 Medium (respectively) supplemented with 10% fetal bovine serum. Cells were kept in a humidified tissue culture incubator at 37°C in 5% CO₂. Calcium phosphate transfections were carried on cells at approximately 40% confluency, followed by a change in media approximately 24 hours from time of transfection. siRNA transfections were conducted using Lipofectamine RNAi max (Invitrogen) according to the manufacturer’s protocol. DNA transfection of H1299 and A549 cells was conducted with Lipofectamine 2000 (Invitrogen) or Lipofectamine LTX (Invitrogen) respectively, according to the manufacturer’s protocol.

3.3.3 Protein Concentrations

Protein concentrations were determined using the Lowry method (Fisher).

3.3.4 Immunoblotting and Immunoprecipitation

Cells were lysed (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors) and centrifuged at 14,000 rpm at 4 °C for 10 min. Aliquots of supernatants were collected for analysis of total protein concentration. For immunoprecipitation, equal amounts of remaining cell lysates were incubated with primary antibody, followed by incubation with protein G-Sepharose beads. The precipitates were washed three times with lysis buffer, eluted with sample buffer, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose followed by blocking in 5% skim milk, and incubation with primary antibody in TBST overnight at 4°C. Following incubation with HRP conjugated secondary antibody, proteins were visualized using West Dura Super Signal ECL (Fisher) and imaged on a VersaDoc Imaging system (BioRad).
3.3.5 Scratch Assays and Polarization Assay

To assess cell migration, a confluent monolayer of cells was scratched with a sterile pipette tip to create an opening, or “wound”. Following wounding, cells were incubated in serum containing medium for 4 hours to allow for cell polarization and leading edge formation. Cells were then fixed and processed for Immunofluorescence microscopy. For the polarization assay, A549 cells were transfected with control or aPKC directed siRNA. 24 hours post transfection, cells were seeded subconfluently onto coverslips to allow cells to establish front-rear polarization. 24 hours following seeding, cells were fixed and processed for immunofluorescence microscopy. For quantification of polarization, immunofluorescence images were acquired and scored for polarization phenotype. At least 100 cells were assessed per condition, per experiment. Graphs represent the average of 3 independent experiments ±SD.

3.3.6 Immunofluorescence Microscopy

For immunofluorescence microscopy studies, cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and incubated for 1 hour in blocking solution (10% FBS/PBS) then incubated with the indicated primary antibodies in blocking solution. Following incubation with the appropriate Cy-conjugated secondary antibodies (or streptavidin), and A555 Phalloidin, cells were visualized by immunofluorescence microscopy using an inverted IX81 Microscope (Olympus, Canada).

3.3.7 Transwell Migration Assays

H1299 cells were transfected with equal amounts of either empty vector (pIRES) or one of the Par6 constructs in the pIRES vector. Cells were serum starved for 3 hours prior to being seeded onto the top of a transwell chamber (Costar). 30 000 cells per condition were seeded and were allowed to migrate towards medium containing 10% serum. In parallel, 30 000 cells from each condition was seeded onto coverslips in
medium containing 10% serum. After 18 hours, cells on the topside of the transwell membrane were removed with a cotton swab, and the migrated cells (on the underside of the transwell) were fixed with 4% paraformaldehyde. Cells on both transwell and the coverslips were stained with DAPI and mounted onto glass slides. Images were acquired using an IX81 inverted microscope (Olympus). Ten representative fields were acquired at 200x magnification and quantified. Graphs represent the average of 3 independent experiments. Cells expressing GFP were counted for each transwell. Cells that were plated simultaneously on coverslips were also quantified and were compared to DAPI stained cells (total cells) to determine transfection efficiencies.

3.3.8 EMT and Migration

A549 cells were transfected with either control or aPKC directed siRNA. 24 hours post transfection, cells were serum starved for 3 hours prior to treatment. Cells were then incubated in serum free medium containing none, or 250 pM TGFβ for 0, 48, or 72 hours to induce EMT. At each time point, cells were brightfield imaged using a IX71 inverted microscope (Olympus), processed for immunofluorescence microscopy, or processed for immunoblotting. For quantification of stress fibre formation, immunofluorescence images (multiple fields) were acquired and assessed for F-Actin morphology over 3 independent experiments. At least 100 cells were assessed per condition, per experiment (> 600 cells total per experiment were assessed). “Cortical” was defined as cells showing F-actin staining to the outer regions (membrane) with no F-Actin fibres through the middle of the cell; “Intermediate” was defined as cells that still showed some cortical staining, but had several F-actin fibres spanning through the middle of the cell (1-5); “Elongated” was defined as cells that showed little cortical staining, and >5 stress fibres spanning through the middle of the cell. Graphs represent the average of 3 independent experiments.

For cell migration analysis, transfected A549 cells were serum starved and treated with TGFβ for 48 hours. After 48 hours, 30 000 cells were seeded into transwell chambers. Cells were allowed to migrate towards serum free medium, or medium containing 10% FBS. After 18 hours, transwell chambers were processed as described
above. Cell nuclei on the underside of the membrane were visualized and counted using DAPI staining. Ten representative fields were acquired at 200x magnification and quantified. Graphs represent the average of 4 independent experiments normalized to the serum free control. Cells that were plated simultaneously into a 6 well culture dish were lysed after 18 hours and processed for SDS-PAGE to assess E-cadherin loss, and subsequently whether EMT had occurred.

3.3.9 Reverse Transcription, Real time PCR and Statistical Analyses

Total RNA was isolated from A549 cells using the RNAeasy Mini kit (Qiagen) according to the manufacturer’s protocol. For complementary DNA (cDNA) synthesis, 1000 ng of total RNA was reverse transcribed using the Superscript® VILO cDNA synthesis kit according to manufacturer’s protocol. (Applied Biosystems). A cDNA equivalent to 10 ng of total RNA was used for all PCR reactions in a total volume of 20 μl. Each reaction was carried out in triplicate. Quantitative PCR (qPCR) reactions were conducted using SsoFast™ EvaGreen® supermix (BioRad) using a Chromo4 Real-time Thermal Cycler (Bio-Rad) according to the recommended protocol of the manufacturer. Primer sequences (5'-3') are as follows: PKCα (TACGGCAGGAGATACAACC and TCGGAGCTCCCAACAATATC), PKCζ (ATCATTCATGTTTTTCCCGAGCA and GTTGGGCACGGTACAGCTTC), SNAI-1 (AATCGGAAGCCTAACTACAGCG and GTCCCAGATGAGCATTGGCA), SNAI-2 (ATACCACAACCAGAGATCCTCA and GACTCACTCGCCCCAAGATG), and Beta-Actin (GGGAAATCGTGCGTGACATTAAG and TGTGTGGGCGTACAGGTCTTTG), and POLR2A (GGATGACCTGACTCATCTCCAAGATG and CGGAAAAATCAGGTGGAGGCC). Primers were selected using Primer3 [38] as well as PrimerBank [39-41]. Baseline and threshold for Ct calculation were set manually using the Opticon Monitor 3.1 Software (Bio-Rad). PCR efficiencies (E) were calculated using cDNA dilution curves and were > 90% for all genes assessed. Calculated PCR efficiencies were used for gene expression quantification using the Pfaffl formula[42], ratio = \((E_{target})^{ΔCt\ target(\ control-tREATED)} / (E_{reference})^{ΔCt\ ref(\ control-tREATED)}\), where control = siControl, no TGFβ. Final ratios were calculated using
geometric averaging [43] from two reference genes: POLR2A, a gene which was found to be a suitable reference gene in NSCLC models [44], and β-Actin. Gene expression of each treatment is expressed in relation to the control (siControl, no TGFβ) and is an average of 3 independent experimental trials. One-way ANOVA analysis followed by post-hoc Tukey’s Tests was used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism® Software 5.0 and p values of <0.05 were considered statistically significant.

3.3.10 Site Directed Mutagenesis

Lysine (K) 19 and Serine (S) 345 were mutated to alanine (A) and glutamic acid (E) respectively using the Quickchange mutagenesis kit (Agilent Technologies) onto the Par6-Flag-pCMV5b template according to manufacturer guidelines. Both mutants were transformed into a XL1 Blue strain of *Escherichia coli*, amplified, purified by the Qiagen Miniprep Kit (Qiagen), and sequenced at the London Regional Genomics Centre (London, ON, Canada).

3.3.11 Statistical Analysis

One-way or Two-way ANOVA analysis followed by post-hoc Bonferroni’s Tests were used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism® Software 5.0 and p values of < 0.05 were considered statistically significant.

3.4 Results

3.4.1 TGFβ receptors and aPKCζ localize to the leading edge of migrating cells

Migrating fibroblasts are polarized and show a clear leading edge as they migrate towards a wound. The TGFβ receptors as well as the atypical PKCζ have been shown to localize to the leading edge of migrating cells [45]. Furthermore, PKCζ, TGFβ and Par6
have been shown to be involved in membrane protrusion dynamics through Smurf1 [12, 25]. Here we investigated whether PKC\(\tau\), the other member of the aPKC family, co-localizes with TGF\(\beta\) receptors at the leading edge and membrane protrusions of migrating cells. To do this we carried out scratch assays followed by immunofluorescence microscopy (Figure 3.1). Polarized Rat2 fibroblasts were immunostained for PKC\(\tau\), and Rac1, a leading edge marker, and TGF\(\beta\) receptors, using biotin-labelled TGF\(\beta\) ligand. We observed that the TGF\(\beta\) receptors and PKC\(\tau\) co-localized with Rac1 at the leading edge of migrating fibroblasts (Figure 3.1A), whereas GM130, a marker for the Golgi apparatus, was not detected at the leading edge with PKC\(\tau\) and the TGF\(\beta\) receptors (Figure 3.1B). This co-localization was also observed in polarized A549 adenocarcinoma cells (Figure 3.9B, please see below). Having ascertained that aPKC and TGF\(\beta\) receptors co-localized in migrating cells, we next sought to determine whether there was an interaction between PKC\(\tau\) and the TGF\(\beta\) receptors.
Figure 3.1
Figure 3.1. Atypical PKCι co-localizes with TGFβ receptors at the leading edge of migrating cells

Rat2 fibroblasts were scratched and incubated for 4 hours to establish cell polarity and then fixed, permeabilized and immunostained with anti-PKCι (PKCι, blue), biotin-labelled TGFβ (biotin-TGFβ; red), anti-Rac1 (Rac1, green; A) or anti-GM130 (Golgi apparatus marker, green; B). The direction of cell migration is indicated with the white arrows and PKCι, Rac1 and TGFβ receptors at the leading edge of migrating cells are indicated by blue, green and red arrowheads, respectively. The white arrowheads indicate the co-localization of all three proteins. Shown are representative images from at least 3 independent replicate experiments. Bar = 10 μm.
3.4.2 TGFβ receptors and PKCζ interact via Par6

Previous work has shown that PKCζ interacts with TGFβ receptors through an association with Par6 [10]. Par6 and aPKC each contain a distinct PB1 (Phox-BEM1) domain through which they interact [46]. Par6 is also known to be an adaptor protein linking several kinases and small GTPases to facilitate cellular processes such as cell polarization and migration [16, 17, 24, 26, 47]. To determine if PKCζ interacted with TGFβ receptors, we expressed TGFβ receptors in the presence of wild-type Par6 or a mutant of Par6 that lacks the PB1 domain (Par6-ΔPB1) and PKCζ in HEK293T cells. Following immunoprecipitation of TβRII and immunoblot analyses, we observed that PKCζ interacted with TGFβ receptors. Furthermore, this association occurred in the presence of wild-type Par6 but not in the presence of the mutant of Par6 that lacks the PB1 domain (Figure 3.2, lanes 9 and 10). This result suggests that there is a complex formation between the TGFβ receptors and aPKC via Par6.
Figure 3.2. Atypical PKCι associates with TGFβ receptors through Par6

HEK293T cells expressing the indicated combinations of GFP-tagged PKCι, HA-tagged TGFβ type II receptors (TβRII), Flag-tagged TGFβ type I receptors (TβRI), wild type Par6 (Par6 WT) or a mutant of Par6 that does not associate with TGFβ receptor (Par6-ΔPB1) were lysed and immunoprecipitated (IP) with anti-HA antibodies. The immunoprecipitates were then subjected to SDS-PAGE, and immunoblotted with anti-(α)-GFP, α-HA or α-Flag antibodies as primary antibodies to visualize proteins that co-precipitated with TβRII (top panel). The non-specific immunoglobulin heavy chain is indicated (IgG) and cell lysates are shown in the bottom panel. Shown are representative immunoblots from at least 3 independent replicate experiments.
3.4.3 aPKC phosphorylates Par6

Phosphorylation of Par6 by TβRII on S345 is an important step in TGFβ-dependent epithelial to mesenchymal transition (EMT) [10, 12]. Given the oncogenic role of aPKC in various cancers, and after seeing that aPKC and TGFβ receptors co-localized and interacted through Par6, we assessed whether aPKC had an effect on phospho-Par6 levels (P-Par6). We used immunoblotting to determine the levels of S345 phosphorylated Par6 in the presence of receptors and aPKC isoforms (Figure 3.3).

We first expressed TGFβ receptors and Par6 in HEK293T cells and assessed P-Par6 levels in the presence of TGFβ receptors (Figure 3.3A). As expected, co-expression of TGFβ receptors and Par6 resulted in Par6 phosphorylation. We next determined whether co-expression of aPKC would alter P-Par6 levels by co-expressing TGFβ receptors, wild type Par6, a Par6 mutant that does not bind aPKC (Par6 K19A), and/or aPKC isoforms (Figure 3.3B). Expression of Par6 with TGFβ receptors resulted in an increase in P-Par6 levels and this was greatly increased in the presence of either PKCα or PKCζ (Figure 3.3B, lanes 2, 4 and 7). Interestingly, when we co-expressed the receptors, aPKC and a mutant of Par6 that does not interact with aPKC Par6 (K19A), we observed Par6 phosphorylation levels seen with TGFβ receptors alone (Figure 3.3B, lanes 5 and 8). This indicated that aPKC enhanced Par6 phosphorylation when co-expressed with receptors, however, the possibility existed that aPKC could phosphorylate Par6 independent from the TGFβ receptor (diagrammed in Figure 3.3C). We explored this idea next.

3.4.4 aPKC phosphorylates Par6 independent of TGFβ receptors

To further assess if aPKC could induce the phosphorylation of Par6 in the absence of TGFβ receptors, we expressed Par6 in the presence or absence of wild-type or kinase deficient aPKC, in the absence of exogenous TGFβ receptor expression (Figure 3.4A). We observed that active aPKC phosphorylated Par6 but the kinase deficient aPKC did not (Figure 3.4A). To assess if the physical interaction of aPKC and Par6 is necessary for the phosphorylation of Par6, we conducted an immunoprecipitation experiment in cells...
expressing the indicated WT or mutant constructs (Figure 3.4B). We observed that both the wild type and kinase deficient PKCζ associated with Par6, but only active PKCζ increased P-Par6 levels (Figure 3.4B). In addition, we utilized a mutant of Par6 (Par6-K19A) that does not interact with aPKC due to the mutation of a lysine residue in the PB1 domain of Par6 [22, 48, 49]. Accordingly, we observed that both active and kinase deficient PKCζ do not associate with Par6-K19A, and correspondingly, we observed very little Par6 phosphorylation. Finally, when we co-expressed Flag-Par6 and each aPKC in A549 NSCLC cells we also observed that both aPKCs increased phosphorylated Par6 levels indicating that the above results were not cell type specific (Figure 3.4C). These results show that in addition to TGFβ receptors, Par6 can be phosphorylated by aPKC. Furthermore, this phosphorylation is dependent on both aPKC-Par6 association and aPKC kinase activity. These results are summarized in a diagram in Figure 3.4D.
Figure 3.3
Figure 3.3. aPKCs associate and phosphorylate Par6 on S345

(A) TGFβ Receptors phosphorylate Par6 on S345. HEK 293T cells expressing the indicated combinations of HA-tagged TGFβ type II receptors (TβRII), Flag-tagged TGFβ type I receptors (TβRI) or Par6 (Par6) were lysed and immunoblotted with anti (α)-HA, α-Flag or α-Phospho-specific S345 Par6 (α-P-Par6) antibodies. The relative mobility of each protein is indicated on the left. Shown are representative immunoblots from at least 3 independent replicate experiments.

(B) aPKC isoforms enhance TGFβ receptor phosphorylation of Par6. HEK293T cells expressing the indicated combinations of GFP-tagged PKCλ, HA-tagged PKCζ or TGFβ type II receptors (TβRII), Flag-tagged TGFβ type I receptors (TβRI), wild type Par6 (Par6 WT) or a mutant of Par6 that does not associate with aPKC (K19A) were lysed, subjected to SDS-PAGE, and immunoblotted with anti (α) -GFP, α-PKCζ, α-Flag, α-Par6 or α-P-Par6 antibodies. Shown are representative immunoblots from at least 3 independent replicate experiments.

(C) A summary of the data are diagrammed in C. (1): aPKC can increase Par6 phosphorylation alongside TGFβ receptors, but also, potentially independent of the TGFβ receptors (2).
Figure 3.4
Figure 3.4. aPKC can phosphorylate Par6 independent of TGFβ receptors

(A) Par6 phosphorylation is dependent on aPKC activity. HEK293T cells expressing the indicated combinations of HA-tagged wild type (WT) or kinase deficient (KR) PKCζ and Flag-tagged Par6 (Par6) were lysed, subjected to SDS-PAGE and immunoblotted with anti (α)-PKCζ, α-Par6 or α-P-Par6 antibodies. Shown are representative immunoblots from at least 3 independent replicate experiments.

(B) Par6 phosphorylation is dependent on aPKC association. HEK293T cells expressing HA-tagged wild type (WT) or kinase deficient (KR) PKCζ and Flag-tagged Par6 (Par6) or a mutant of Par6 that does not associate with aPKC (K19) were lysed and immunoprecipitated (IP) with anti-Flag antibodies. The immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti (α)-PKCζ, or α-Par6 antibodies (top panel). Cell lysates were immunoblotted with the antibodies indicated to assess protein expression and Par6 phosphorylation (bottom panel). Shown are representative immunoblots from at least 3 independent replicate experiments.

(C) Par6 phosphorylation occurs in A549 cells. A549 cells transfected with the indicated combinations of Flag-Par6, GFP-PKCλ, and HA-PKCζ were lysed, processed for SDS-PAGE, and immunoblotted for anti(α)-GFP, α-HA, α-Flag, α-Actin and α-P-Par6 as indicated. A non-specific band in the P-Par6 blot is indicated by an asterisk. Shown are representative immunoblots from at least 3 independent replicate experiments.

(D) A summary of the data are diagrammed in D. aPKC can phosphorylate Par6 in the absence of TGFβ receptors. The kinase activity of aPKC and aPKC binding ability to Par6 are important for this phosphorylation.
3.4.5 Par6 acts as a scaffold between aPKC and TGFβ receptors

We next set out to determine whether increased expression of aPKC would affect the association of Par6 with type I TGFβ receptors (TβRI; Figure 3.5A). TβRI and Par6 constructs (WT or K19A) were expressed in the presence of increasing levels of aPKC. Par6 protein was then immunoprecipitated and immunoblotted for aPKC and TβRI to assess association with these binding partners. We found that increasing aPKC expression reduced the amount of TβRI bound to Par6, accompanied by an increased association of aPKC with Par6 (Figure 3.5A). However, the Par6 mutant that cannot associate with aPKC (K19A) continued to associate with TβRI despite increasing aPKC levels (Figure 3.5B, right panel). Interestingly, we also observed that the steady state levels of Par6 increased when it was displaced from the TβRI (Figure 3.5A; cell lysates, lanes 4-6). However, this was not observed in cells expressing Par6 K19A (Figure 3.5A; cell lysates, lanes 8-10).

The ubiquitin ligase Smurf1 is known to regulate the levels of Par6 as well as the TGFβ receptors [50-52]. Interestingly, we observed that expression of Par6 with TGFβ receptors and Smurf1 decreased the steady state levels of receptors, Par6 and Smurf1 (Figure 3.5B). This effect was reduced when the K19A or S345A mutants were expressed in place of WT Par6, suggesting that both endogenous aPKC association with Par6 and phosphorylation on S345 may be involved in Smurf1 mediated degradation of the TGFβ receptor-Par6 complex.

These data further substantiate the scaffolding of TβRI and aPKC via Par6 and implicates aPKC in the degradation of receptor associated Par6. Taken together, our results suggest that aPKC association and S345 phosphorylation of Par6 can regulate TGFβ receptor and Par6 levels.
Figure 3.5
Figure 3.5. aPKC reduces TβRI associated Par6

(A) aPKC displaces Par6 from TGFβ Receptors. HEK293T cells were transfected with Flag-TβRI, Flag-Par6 (WT or K19A) and increasing amounts of GFP-tagged PKC. Lysates were immunoprecipitated (IP) with anti-Par6 antibodies. The immunoprecipitates (IP) were subjected to SDS-PAGE, and immunoblotted anti (α)-GFP, or α-Flag antibodies as primary antibodies to visualize proteins that co-precipitated with Par6. Total lysates are shown in the bottom panel. Par6-associated TβRI levels were quantitated and are shown graphically. Shown are representative immunoblots from 3 independent replicate experiments. (n=3 ± SEM, Two-way ANOVA, *p<0.05).

(B) Par6 mutants attenuate aPKC-dependent reduction of TGFβ Receptor steady state levels. HEK 293T cells expressing HA-tagged TGFβ type II receptors (TβRII), Flag-tagged TGFβ type I receptors (TβRI), Flag-tagged Smurf1, and Flag-tagged Par6 (WT, K19A, or S345A) were lysed, subjected to SDS-PAGE and immunoblotted with anti α-HA, α-Flag, α-Actin, or α-Phospho-specific S345 Par6 (α-P-Par6) antibodies. Shown are representative immunoblots from at least 3 independent replicate experiments.
3.4.6 aPKC expression and association increases Par6 levels

After observing that the expression of aPKC displaced Par6 from TβRI and increased steady state Par6 levels, we assessed the protein stability of Par6 when co-expressed with PKCθ (Figure 3.6A). Using the translation inhibitor, cycloheximide, cells expressing Par6 were assessed for Par6 levels over a 6 hour time course. We observed that Par6 levels dropped to approximately 16.2% ± 8.3% within 6 hours (Figure 3.6A, right panel). When Par6 was co-expressed with aPKC, we observed stabilization of Par6 protein levels. Indeed, when Par6 was expressed with aPKC, Par6 protein levels remained 88.8% ± 3.9% of the Time 0 control (Figure 3.6A, right panel).

We next assessed the effect of Smurf1 on Par6 levels. We observed that the co-expression of Par6 with Smurf1 led to reduced steady state levels of Par6 (Figure 3.6B). However, co-expression of aPKC with Par6 blocked this degradation, as observed by an increase in steady state levels of Par6 (Figure 3.6B). We further wanted to explore whether S345 phosphorylation of Par6 would alter its steady state levels. We analyzed this by expressing aPKC in cells expressing Par6 or the K19A and S345A mutants (Figure 3.6C). Notably, we observed an increase in steady state Par6 levels when aPKC was introduced to cells expressing WT or S345A Par6 mutant (Figure 3.6C, lanes 2 to 3 and lanes 6 to 7). This effect was not observed in cells expressing Par6-K19A, a mutant that cannot associate with aPKC (Figure 3.6B, lane 4 vs. 5). These results suggest that aPKC association and not the phosphorylation of Par6 inhibited Smurf1-mediated reduction of steady state levels of Par6 (Figure 3.6C).
Figure 3.6
Figure 3.6. aPKC expression stabilizes Par6 protein levels

(A) HEK 293T cells were transfected with Flag-Par6 and empty vector or with Flag-Par6 and GFP tagged PKC\(\alpha\). These cells were then subjected to a time course with the translation inhibitor cycloheximide (100 \(\mu\)g/mL) and lysed after the indicated time points. Lysates were subjected to SDS-PAGE, followed by immunoblotting with \(\alpha\)-GFP, \(\alpha\)-Flag, or \(\alpha\)-GAPDH (loading control). Average Par6 levels over time were quantitated and are shown graphically in the right panel. Shown are representative immunoblots from 3 independent replicate experiments. (n=3 ± SEM, Two-way ANOVA, **p<0.01).

(B) HEK 293T cells expressing combinations of Flag-Smurf1, GFP-PKC\(\alpha\), and Flag-Par6, were subjected to SDS-PAGE, followed by immunoblotting with \(\alpha\)-GFP, \(\alpha\)-Flag, or \(\alpha\)-P-Par6 to assess protein or phosphoprotein levels. Shown are representative immunoblots from at least 3 independent replicate experiments.

(C) HEK 293T cells expressing combinations of Flag-Smurf1, GFP-PKC\(\alpha\), and Flag-Par6, Flag-Par6-K19A, or Flag-Par6S345A (phosphorylation site mutated) were lysed and subjected to SDS-PAGE, followed by immunoblotting with the primary antibodies indicated on the right of the panels. Par6 protein levels were quantitated and shown graphically. Shown are representative immunoblots from 6 independent replicate experiments. (n=6, ± SEM).
3.4.7 Par6 phosphorylation and aPKC association are important for Par6 induced migration

Expression and phosphorylation of Par6 have been shown to increase the migration and metastatic processes of breast cancer cells [12]. We therefore tested whether Par6 phosphorylation and aPKC association could affect cell migration of a metastatic NSCLC cell line (H1299) using transwell migration assays (Figure 3.7). Briefly, H1299 cells were transiently transfected with empty pIRES vector, wild type Par6 or the two mutant Par6 constructs (K19A, S345A). Relative expression levels are shown in Figure 3.7A. Our results indicated that overexpression of wild type Par6 increased cell migration (5.3 ± 0.1 fold) towards serum compared to control cells. Cells expressing the aPKC binding mutant of Par6 (Par6-K19A) also stimulated H1299 cell migration by 2.4 ± 0.4 fold. This muted, yet significant stimulation of cell migration was expected, as Par6-K19A can still associate with TGFβ receptors and be phosphorylated by TβRII on S345. Finally, cells expressing the S345 mutant of Par6 that cannot be phosphorylated by either TβRII or aPKC did not significantly stimulate cell migration (Figure 3.7B).

Taken together, these results suggest that aPKC isoforms play a role in NSCLC cell migration both through Par6 association and S345 phosphorylation. We next explored if siRNA directed towards aPKC would affect TGFβ-dependent EMT.
Figure 3.7
Figure 3.7. Par6 induces cell migration

(A) H1299 cells were transiently transfected with empty vector (pIRES), wild-type Par6 (Par6-WT), Par6-K19A or Par6-S345A constructs. Immunoblots of lysates show relative expression levels of transfected cells.

(B) Cells transfected as described in Panel A were plated into the top chamber of a transwell chamber and allowed to migrate towards serum for 18 hours. Representative images from the migration assay are shown above graphical analysis. Data are presented as a percentage of control (pIRES) and represent the average of 3 independent experiments (n=3 ± SEM, One-way ANOVA, **p<0.01). Bar = 100 µm.
3.4.8 aPKC siRNA alters RhoA levels

The TGFβ-Par6 pathway has been shown to regulate EMT by stimulating cytoskeletal remodelling and cell plasticity. A critical step in this process is TGFβ induced degradation of RhoA by Smurf1 [10]. Interestingly, this was shown to be Smurf1-dependent, as reduction of Smurf1 expression blocked RhoA degradation and EMT [10, 25]. Furthermore, both the phosphorylation of Par6 as well as PKC activity, were shown to be important for the recruitment of Smurf1 and targeting of RhoA for degradation [10, 25]. Since we observed that aPKC co-operate with TGFβ receptors to phosphorylate Par6, we next determined whether aPKC played a role in TGFβ induced RhoA degradation. Using siRNA targeting both aPKC isoforms, we monitored RhoA levels in response to TGFβ in A549 NSCLC cells (Figure 3.8).

Previous work has shown, that upon TGFβ stimulation, RhoA levels are reduced by ~20%, followed by the cell cytoskeletal and morphological changes associated with EMT [10]. Similarly, we detected a consistent reduction in RhoA levels following TGFβ treatment in cells transfected with control siRNA (Figure 3.8). However, siRNA targeting aPKC resulted in a TGFβ-dependent increase in RhoA steady state levels (Figure 3.8). Intriguingly, this result was similar to previous findings that RhoA accumulates with Smurf1 knockdown [25]. We next monitored activated aPKC levels in response to TGFβ treatment.
Figure 3.8. aPKC silencing attenuates TGFβ-dependent decreases in RhoA levels

A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were treated with TGFβ followed by lysing and processing for SDS-PAGE and immunoblotting with anti α-RhoA, α-PKCζ, α-PKCι or α-actin antibodies. Average RhoA levels were quantitated and graphed in the bottom panel. Shown are representative immunoblots from 5 independent replicate experiments. (n=5 ± SEM, Two-way ANOVA, *p<0.05).
3.4.9 TGFβ treatment activates aPKC

We next examined aPKC activity in response to TGFβ using a phosho-specific antibody that detects T410 phosphorylated PKCζ (or T403 in PKCθ). Phosphorylation of aPKC on this site is known to regulate its enzymatic activity [53]. Consistent with studies carried out using prostate cancer cells and mouse embryonic fibroblasts [24], we observed that TGFβ treatment increased the levels of endogenous phospho-PKCθ/ζ in A549 cells (Figure 3.9, Cell lysates). Interestingly, expression of Par6 also increased P-PKCθ/ζ levels to equivalent levels seen with TGFβ addition, indicating that Par6 may also scaffold aPKC to endogenous activators. Following immunoprecipitation of Par6 and immunoblotting for activated aPKC, we found that Par6-associated P-PKCθ/ζ levels increased by 22 ± 7% upon TGFβ treatment compared to control (Figure 3.9, IP and Graph). These results prompted us to assess whether aPKC played a role in TGFβ induced cell morphology and EMT.
Figure 3.9. TGFβ increases total and Par6 associated aPKC activity

A549 cells transfected with empty vector or Flag-tagged Par6 were treated with (or without) TGFβ for 1 hour. Cell lysates were immunoprecipitated (IP) with anti-Flag antibodies. The immunoprecipitates (IP) were subjected to SDS-PAGE, and immunoblotted with anti (α)-phospho-specific PKCζ (P-PKCζ), total PKCζ, and α-Flag as indicated. The asterisk (*) denotes a non-specific band. Average Par6 associated P-PKCζ levels were quantitated and are shown graphically below immunoblots. Shown are representative immunoblots from 3 independent replicate experiments. (n=3 ± SEM).
3.4.10 aPKC siRNA induces changes in cell morphology

TGFβ-treated A549 cells acquire a spindle–shaped appearance and reduce cell-cell contacts [7]. We observed that siRNA-mediated aPKC silencing reduced TGFβ induced morphological changes of A549 cells, as assessed by DIC microscopy (Figure 3.10A). In cells expressing aPKC isoforms, the cobblestone appearance of A549 cells became elongated in the presence of TGFβ after 48 and 72 hours. In contrast, aPKC silenced cells retained their cobblestone morphology with TGFβ treatment at both time points. We also examined the front-rear polarization of sub-confluent control and aPKC silenced A549 cells (Figure 3.10B). Interestingly, we found that the proportion of cells that polarize and form a leading edge is reduced in aPKC silenced cells. Furthermore, we detected a greater number of cells with a rounded phenotype in aPKC-silenced cells than in control (Figure 3.10C). This is in agreement with previous work that aPKC plays an important role in cell polarization [21] and similar to work that cells are less protrusive with Smurf1 knockdown [25].
Figure 3.10
Figure 3.10. aPKC silencing reduces TGFβ induced changes in cell morphology

(A) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were treated with TGFβ for 0, 48, or 72 hours and imaged by brightfield microscopy. Shown are representative images from at least 3 independent replicate experiments. Bar = 100 μm.

(B) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were plated sub-confluently, fixed, permeabilized and immunostained with anti-PKCι (PKCι, blue) anti-Rac1 (Rac1, green), and biotin-labelled TGFβ (biotin-TGFβ; red). Shown are representative images from 3 independent replicate experiments.

(C) The average number of total elongated verses rounded cells was quantitated over 3 experiments and is presented graphically (n=3 ± SEM, Two-way ANOVA, **p<0.01). Bar = 100 μm.
3.4.11 aPKC siRNA reduces TGFβ induced EMT

Since we observed that both aPKCs and TGFβ receptors could phosphorylate Par6, that TGFβ increases activated aPKC levels, and that aPKC modulates TGFβ induced RhoA degradation, we next determined to what extent aPKC was involved in TGFβ induced EMT. TGFβ-dependent EMT involves the loss, in the expression and organization, of the adhesion protein E-cadherin [1, 2, 7] and leads to decreased cell adhesion and increased cell motility.

Using siRNA directed at both aPKC isoforms, we assessed changes in TGFβ induced E-cadherin protein levels in A549 NSCLC cells (Figure 3.11A). Control, or aPKC-silenced cells were treated with TGFβ for 0-72 hours and cell lysates were immunoblotted for E-cadherin, P-Smad2, PKCδ, PKCζ, Smad2 and Actin. We observed that E-cadherin protein levels were significantly reduced in control cells incubated with TGFβ after 48 or 72 hours (Figure 3.11A, lanes 1-3). In addition, we observed Smad2 phosphorylation after 48 and 72 hours of TGFβ treatment. In contrast, basal E-cadherin levels were significantly higher in aPKC-silenced cells and were only moderately decreased in response to TGFβ (Figure 3.11A, lanes 4-6). This suggested that aPKC was important for TGFβ induced E-cadherin loss despite efficient phosphorylation of Smad2. Interestingly, the levels of mRNA for the transcriptional repressors of E-cadherin, SNAI1 and SNAI2, both increased to a similar extent in control and aPKC silenced cells (Figure 3.11B). This is consistent with the idea that Smad2 signalling was still occurring in both control and aPKC silenced cells.

We next assessed E-cadherin expression and stress fibre formation in A549 cells by immunofluorescence microscopy. We observed that in the absence of TGFβ treatment, 88 ± 5% of control cells and 82 ± 4% of siPKCδ/ζ cells contained few spanning stress fibres and maintain a cortical F-actin distribution (Figure 3.11C). In contrast, upon TGFβ treatment, the majority of cells transfected with control siRNA (76 ± 4%) display elongated, cell spanning stress fibres after 48 and 72 hours. This effect was significantly reduced in aPKC-silenced cells in which only 15 ± 5% of cells show elongated stress fibres (Figure 3.11C). Furthermore, TGFβ induced E-cadherin loss was
reduced in aPKC silenced cells (Figure 3.11C), consistent with immunoblot analyses (Figure 3.11A). Taken together, these data suggest that aPKC is necessary for efficient TGFβ induced EMT of NSCLC cells.

After observing that aPKC knockdown attenuated TGFβ induced EMT, we next assessed cell motility. Our results showed that after 48 hours of TGFβ stimulation, siControl cells migrated towards serum to a greater extent than aPKC-silenced cells, as assessed by transwell migration assays (Figure 3.11D). This indicated that aPKC silenced cells that did not undergo EMT were also less motile.

3.4.12 aPKC knockdown reduces claudin loss and individual aPKC siRNA reduces TGFβ induced EMT

We also examined levels of a second epithelial marker, Claudin 1, in cells transfected with control siRNA or siRNA targeting both aPKC isoforms. Similar to E-cadherin levels, TGFβ induced a loss in Claudin1 levels in control cells, but this effect was abrogated in aPKC knockdown cells (Figure 3.12A). Furthermore, we wanted to examine whether individual aPKCs were involved in reducing TGFβ induced E-cadherin loss. Using siRNA directed at each aPKC isoform individually as well as both aPKC isoforms together, we assessed changes in TGFβ induced E-cadherin protein levels in A549 NSCLC cells. Interestingly, knockdown of individual aPKCs (siPKC α, or siPKC ζ,) could also reduce TGFβ induced E-cadherin loss, although only cells transfected with siPKCα showed statistically significant results through average densitometrical analysis (Figure 3.12B). Importantly, the double knockdown (siPKC α/ζ) also significantly reduced TGFβ induced E-cadherin loss as observed before (Figure 3.12B). Taken together, these results indicated that aPKC knockdown cells were indeed exhibiting a greater degree of epithelial phenotype when stimulated with TGFβ. We next assessed whether re-introduction of exogenous P-Par6 into aPKC silenced cells could restore TGFβ induced EMT.
Figure 3.11
Figure 3.11. aPKC silencing reduces TGFβ induced EMT and motility

(A) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were treated with TGFβ for 0, 48, or 72 hours. Cells were then lysed and processed for SDS-PAGE and immunoblotting with anti (α)-E-cadherin, α-phospho-specific Smad 2 (α-P-Smad2), α-Smad2, α-PKCζ, α-PKCι or α-actin antibodies. The asterisk (*) denotes a non-specific band. Shown are representative immunoblots from at 5 independent replicate experiments. Average E-cadherin levels were quantitated and graphed (bottom panel, n=5 ± SEM, Two-way ANOVA, **p<0.01).

(B) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were treated with or without TGFβ. After 24 hours, total RNA was extracted; reverse transcribed, and subjected to quantitative real time PCR analysis (qPCR) of PRKCI (PKCι), PRKCZ (PKCζ), SNAI1 (Snail) and SNAI2 (Slug). Average relative gene expression from 3 independent experiments is shown graphically (n=3 ± SEM, Two-way ANOVA, p<0.05).

(C) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were treated with or without TGFβ 48 hours followed by processing for immunofluorescence microscopy and stained for filamentous actin with Phalloidin (red), E-cadherin (green) and DAPI to image nuclei (blue). Cell morphology changes over 3 independent experiments were quantified and are displayed graphically below representative images. "a" indicates a statistically significant change with TGFβ treatment. "b" indicates a statistically significant difference between siControl and siPKCι/ζ cells at the indicated time point. (n=3 ± SEM, Two-way ANOVA, *p<0.05). Bar = 100 μm.

(D) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) were untreated, or treated with TGFβ for 48 hours and assessed for migration towards serum for 18 hours. Representative images from the migration assay are shown below the graphical analysis (n=4 ± SEM, Two-way ANOVA, **p<0.01). Bar = 100 μm.
Figure 3.12
Figure 3.12. aPKC silencing reduces TGFβ induced Claudin-1 loss and individual aPKC knockdown also reduces TGFβ induced E-cadherin loss

(A) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKCι/ζ) were treated with or without TGFβ 48 hours. Cells were then lysed and processed for SDS-PAGE and immunoblotting with (α)-Claudin1, or α-Actin antibodies. Shown are representative immunoblots from 3 independent replicate experiments (n=3).

(B) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota (siPKCι), zeta (siPKCζ), or both (siPKCι/ζ) were treated with or without TGFβ for 48 hours. Cells were then lysed and processed for SDS-PAGE and immunoblotting with α-E-cadherin or α-actin antibodies. Three experiments were carried out as described in Panel A, quantitated and are shown graphically below immunoblot (n=3 ± SEM, Two-way ANOVA, *p<0.05)
3.4.13 Phospho-mimetic Par6 induces TGFβ-dependent EMT and RhoA degradation in aPKC silenced cells

We next sought to test whether a reduction in phospho-Par6 was indeed responsible for the reduced EMT response we were seeing in aPKC-silenced cells. To carry this out, we generated a serine 345 phospho-mimetic version of Par6 (Par6 S345E). We first ascertained that the Par6 S345E mutant was phospho-mimetic by immunoblot analysis using the phospho-Par6 antibody (Figure 3.13A). Indeed, the phospho-S345 Par6 antibody recognized the Par6 S345E mutant.

Next, we introduced Par6 S345E into aPKC-silenced cells, and assessed E-cadherin protein levels following TGFβ-treatment (Figure 3.13B). Consistent with observations in Figure 3.11A, aPKC silencing reduced TGFβ-dependent loss of E-cadherin levels (Figure 3.13B, lanes 1-4). However, the Par6 S345E mutant restored the TGFβ effect (lanes 9 and 10). Furthermore, Par6 S345E reduced the induction of basal E-cadherin levels observed in aPKC-silenced cells (Figure 3.13B, lane 3 vs. 9).

As observed with the E-cadherin analysis, expression of the phospho-mimetic Par6 S345E mutant significantly reversed the effect of aPKC silencing on TGFβ-dependent RhoA levels (Figure 3.13C). aPKC silenced cells contained a significant increase of RhoA protein levels in response to TGFβ. However, not only did Par6 S345E reduce this effect, cells expressing this Par6 mutant had lower RhoA levels than cells transfected with only control siRNA (Figure 3.13C, bottom panel).

Taken together, the data suggest that the phosphorylation of Par6 by both TGFβ receptors and aPKC is necessary for efficient EMT of A549 NSCLC cells (summarized in Figure 3.14).
Figure 3.13
Figure 3.13. A S345E phospho-mimetic Par6 mutant restores TGFβ-dependent E-cadherin and RhoA loss in aPKC silenced cells

(A) HEK293T cells transfected with Flag-Par6 (WT, K19A, S345A, or S345E) were lysed, processed for SDS-PAGE and immunoblotted with anti (α)-Flag, or α-Phospho-Par6 antibodies to determine the relative levels of P-Par6. A representative immunoblot from three independent replicate experiments is shown.

(B) A549 cells transfected with control siRNA (siControl) or siRNA targeting PKC iota and zeta (siPKC ι/ζ) and Par6-S345E or empty vector, were incubated in the presence or absence of TGFβ for 48 hours. Cells were then lysed and processed for SDS-PAGE and immunoblotting with anti (α)-E-cadherin, α-PKCζ, α-PKCι or α-Actin antibodies. Average E-cadherin levels from 4 independent replicate experiments were quantitated by densitometrical analysis and graphed below a representative immunoblot (n=4 ± SEM, Two-way ANOVA, *p<0.05).

(C) A549 cells were treated as described in panel B. Cells were then lysed and processed for SDS-PAGE and immunoblotting with anti (α)-RhoA, and α-Actin antibodies. Average RhoA levels from 4 independent replicate experiments were quantitated by densitometrical analysis and graphed below a representative immunoblot (n=4 ± SEM, Two-Way ANOVA *p<0.05).
Figure 3.14 Atypical PKC phosphorylates Par6 and facilitates TGFβ induced EMT

TGFβ stimulation leads to the activation of aPKC. Both aPKC and TGFβ receptors can phosphorylate Par6 on S345 to initiate RhoA degradation, E-cadherin loss and subsequently EMT.
3.5 Discussion

Epithelial to mesenchymal transition is a critical event in tumour progression leading to the disorganization of tissue architecture. The αPKC-Par6 axis is an emerging pathway shown to be important in the stimulation of EMT and progression of metastatic tumours. Phosphorylation of Par6 is associated with tumours that are more invasive and have been correlated with reduced survival in breast cancer patients [12]. Until now, Par6 phosphorylation on Serine 345 has only been reported to occur through TGFβ receptor activation.

In this report, we have observed that both atypical PKC isoforms (ι and ζ) also phosphorylate Par6 on S345 and suggests a role for αPKC in promoting epithelial derived tumour cells into EMT through the Par6 pathway. Coupled with the observations that αPKC shows malignant activity in various cancers [32, 33, 54] and oncogenic potential in lung cancer [28-30, 34], our results suggest that aberrant phosphorylation of Par6 by αPKC may be a major tumour promoting process.

Interestingly, we discovered that αPKC increases steady state Par6 protein levels, which we attribute to a decrease in Smurf1 mediated degradation. A recent report has indicated that protein kinase A (PKA)-dependent phosphorylation of Smurf1 leads to the protection of Par6 and the simultaneous Smurf1 mediated degradation of RhoA [52]. Given our observations, it would be interesting to explore whether αPKC can also phosphorylate Smurf1, thus leading to protection of Par6 from Smurf1 mediated degradation. Furthermore, Smurf1 may target TGFβ receptors and Par6 for degradation via αPKC association and Par6 S345 phosphorylation. Since we have previously described a role for αPKC in TGFβ receptor trafficking and degradation [35], we believe the current results suggest a role for αPKC regulation of TGFβ receptor-Par6 complexes.

Ozdamar et al. have previously shown that Par6 phosphorylation on S345 is important for TGFβ mediated RhoA degradation and adherens junction dissolution [10]. Here, we show that basal E-cadherin levels are significantly higher in αPKC-silenced cells, and furthermore, TGFβ -induced reductions of both RhoA and E-cadherin are severely impaired when αPKC is silenced. This effect was seen despite similar Phospho-Smad2 signalling levels between control and αPKC silenced cells, highlighting the importance of αPKC in the EMT process via the Par6 pathway.
A similar effect has been reported with respect to the ability of the ErbB2 receptor to disrupt tissue architecture of polarized epithelia [22]. Inhibiting the interaction of aPKC and Par6 (with K19A) blocked the ability of ErbB2 to disrupt the acinar organization of breast epithelial cells [22]. Interestingly, activation of the TGFβ-Par6 pathway has also been shown to disrupt acini-like structure formation in normal murine mammary gland (NMuMG) cells [12]. This effect is reduced through expression of the S345A Par6 mutant indicating that phosphorylation of Par6 on S345 is important for this disruption. Given the observations that both aPKC association with Par6 and S345 phosphorylation are important for tissue organization, we believe that aPKC phosphorylation of Par6 may be critical in the disorganization of normal tissue architecture. Interestingly, introduction of the Phospho-mimetic Par6 (S345E) into aPKC silenced cells restored full TGFβ induced E-cadherin loss and RhoA degradation - highlighting an important role for aPKC induced P-Par6 in these processes.

Taken together, we propose that multiple tumour promoting pathways (including TGFβ and ErbB2) may require aPKC to disrupt cell polarity, and that aPKC may be a target for blocking oncogenic signalling pathways that induce tissue disruption during tumour progression. Indeed, we observed that the silencing of aPKC reduces TGFβ induced EMT and migration of A549 adenocarcinoma cells. Furthermore, others have shown that loss of function experiments of PKCт in A549 cells showed reduced invasive activity as well as significantly reduced tumour growth and expansion in vivo in nude mice [30, 34]. aPKC has recently been implicated in various disease states, and several studies have highlighted the oncogenic characteristics of PKCт in NSCLC [28, 30, 31, 54-56], with an elevated level of PKCт expression reported to be correlated with poor outcome in NSCLC patients. Patients with early stage lung cancer and high PKCт expression are more than 10 times likely to perish from the disease than those with low levels of PKCт [56]. A similar trend is evident in patients with increased PKCт DNA copy number and ovarian cancer [33]. Increased PKCт expression is also correlated with increased cyclin E expression in ovarian cancers, and is implicated with increased proliferation, defects in cell polarity, and decreased survival rates [33]. Furthermore, aPKC has been shown to be important in the Par6 induced cell proliferation in breast
epithelial cells, as the K19A Par6 mutant reduces the mitogenic effects of Par6 signalling [23].

We believe that aPKC is an important component of TGFβ activated phosphorylation of Par6, and that silencing of aPKC gene expression blocks TGFβ induced EMT and migration of NSCLC cells. It will be interesting to explore whether the aPKC-Par6 axis is an important pathway in the metastatic progression of NSCLC tumours in vivo.

3.6 Footnotes

We would like to thank Dr. Jeffrey L. Wrana for generously providing the S345 Phospho-specific Par6 antibody. This work was funded by the Canadian Institutes of Health Research (MOP-93625).

3.7 References


Chapter 4

A version of this chapter has been prepared for submission to J. Cell Biol.
Chapter 4

4.1 Chapter Summary

TGFβ signalling regulates many cellular responses including proliferation, EMT, and apoptosis. This wide array of responses occurs through the intricate control of Smad activity as well as other non-Smad pathways including Par6 and MAPK. My previous work demonstrated that aPKCs are important for TGFβ receptor trafficking, degradation, and signalling pathways. Here I analyzed gene changes and responses in A549 lung cancer cells in which aPKC has been silenced using siRNA.

When analyzing the gene responses in aPKC-silenced cells, we observed a dampening of TGFβ response, as assessed by microarray and qPCR, which correlated with a reduction in Smad2 nuclear accumulation in response to TGFβ. Interestingly, we also detected an increase in p38 MAPK phosphorylation in aPKC-silenced cells. Although the enhanced p38 MAPK levels paralleled an increase in apoptotic response, p38 inhibition did not rescue Smad2 nuclear accumulation. p38 MAPK activation in aPKC silenced cells was found to occur downstream of TRAF6, as TRAF6 knockdown abrogated the increased p38 MAPK phosphorylation we observed in aPKC silenced cells. Interestingly, knockdown of aPKC stabilized TRAF6-TGFβ receptor complexes, providing a mechanism for the enhanced p38 activation observed. Finally, in aPKC silenced cells we observed an increase in the expression of the Smad2 cytoplasmic retention factor, SARA, and concomitantly a reduction in Smad2 release upon TGFβ stimulation. Furthermore, this followed a reduction in Smad2-Smad4 complex formation in aPKC-silenced cells. We reason this deregulated SARA-Smad2-Smad4 exchange leads to a reduced nuclear accumulation of R-Smads and reduced transcriptional response.

We conclude that aPKC influences the stability of receptor binding partners (such as TRAF6 and SARA) which can subsequently affect TGFβ signalling and cellular response.
4.2 Introduction

TGFβ signalling controls many cellular processes including proliferation, apoptosis, and EMT. Aberrant TGFβ signalling is a hallmark of several pathological conditions including cancer and fibrosis [1-5]. The canonical TGFβ pathway involves the cell surface binding of TGFβ ligand to TβRII, which then binds and phosphorylates TβRI [6]. Phosphorylation of TβRI leads to its activation, and its ability to transduce intracellular signalling through the phosphorylation of substrate proteins such as the R-Smads, Smad2 and Smad3 [2, 6]. Once phosphorylated, R-Smads accumulate in the nucleus where they act as transcription factors to regulate subsequent TGFβ gene response [2, 6-9]. Entry into the nucleus of R-Smads is facilitated by directly binding the nucleopore complex, binding to Importins (for Smad3), or facilitated by the binding of the common Smad, Smad 4 [8, 10]

Proteins that control the membrane trafficking and endocytosis of TGFβ receptors play a role in regulating the intensity and duration of TGFβ signals. For example, the efficient regulation of Smad signalling can be facilitated by the adaptor protein SARA (Smad anchor for receptor activation). SARA contains a Smad binding domain, as well as a TGFβ receptor complex interacting region, and acts as a bridge, facilitating R-Smad presentation to the activated receptor complex [11, 12]. SARA also contains a phosphatidylinositol 3-phosphate (PI3P) binding FYVE domain, which induces its association with the early endosome, and links receptor endocytosis and trafficking to Smad signal transduction [12, 13]. Once SARA-bound R-Smads are phosphorylated, they dissociate from the SARA-receptor complex, bind to Smad4, and subsequently translocate to and accumulate in the nucleus to regulate transcription [14]. Interestingly, although receptor endocytosis has been reported to be dispensable for the phosphorylation of R-Smads, it has been reported that endocytosis is required for the efficient dissociation of R-Smads from SARA, nuclear accumulation, and subsequent transcriptional response [15]. The precise regulation of transcriptional activity of Smads in the nucleus is important for the proper execution of embryonic development by controlling tissue patterning, normal organ development, and also for controlling cellular growth and apoptotic response in adult tissues [4, 7, 8, 16, 17].
Although it is established that Smads are central regulators of gene response to TGFβ, multiple Smad independent pathways are also initiated upon TGFβ receptor activation [10, 16-19]. TGFβ can activate the mitogen associated protein kinase family (MAPK). There are three principle MAPK proteins: ERK, JNK, and p38 - each of which has a role in the development and progression of cancer [20]. The p38 MAPK pathway downstream of TGFβ has gained considerable interest as a pathway that regulates apoptosis. Briefly, TGFβ receptor activation leads to the recruitment and Lys-63 linked auto-ubiquitination and activation of TRAF6, an E3 ubiquitin ligase. This stimulates a cascade that culminates in the activation of p38 MAPK and ultimately apoptosis in various cell types [21-23]. Interestingly, aPKC isoforms interact with TRAF6 to mediate cytokine signalling [24], but less is known about whether aPKC mediates the TGFβ-p38 MAPK pathway.

We have previously shown that aPKC isoforms can alter TGFβ signalling patterns in NSCLC cells by altering receptor trafficking, degrading specific receptor complexes and by enhancing Par6 dependent phosphorylation [25, 26]. However, we had not examined gene changes on a large scale. Furthermore, we have not examined whether aPKCs alter TGFβ-induced MAPK pathways. The aPKC isoforms, which consist of PKCτ and PKCζ, are a subset of the Protein Kinase C family that are calcium and diacylglycerol (DAG) independent [27]. Importantly, the aPKCs show altered expression and activities in various cancers [28], and PKCτ has been described as an oncogene [29, 30]. Interestingly, aPKC isoforms have been known to play a role in p38 MAPK induced apoptosis, as inhibition or knockdown of aPKC sensitizes glioblastoma cells to chemotherapeutic agents via a p38 dependent mechanism [31].

In this report we examined TGFβ-dependent transcriptional response in aPKC silenced cells by microarray analyses, and also examined how knockdown of aPKC alters Smad dynamics and MAPK pathways to alter cellular apoptosis.
4.3 Materials and Methods

4.3.1 Antibodies and Reagents

Primary antibodies were purchased from the following vendors: Anti-β-Actin (Sigma-A2668), anti-PKCα (BD Transduction-610175), anti-PKCζ (Cell Signalling Tech-9372), anti-Phospho-Smad2 (Cell Signalling Tech-3101), anti-Smad2/3 (BD Trans-610842), anti-Tubulin (Sigma-T4026), anti H3-Histone (Millipore-05-499), anti-phospho-p38 (Cell Signalling-9211), anti p38 (Cell Signalling- 9212), anti phospho-ERK (Cell Signalling – 4370), anti-phospho-JNK (Cell Signalling Tech-9255s), anti-Smad4 (Abcam-AB40759), anti-SARA (Santa Cruz, sc-9135), anti-Flag (Sigma Aldrich – F3165), anti-Traf6 (Cell Signalling Tech- 8028s), anti-EEA1 (BD Transduction-610457). HRP conjugated secondary goat-anti-rabbit (Thermo Scientific -31460) and goat-anti-mouse (Thermo Scientific -31430) were used for immunoblot analysis. Fluorescently conjugated donkey α-mouse (Life Technologies A21206), donkey α-rabbit (Life Technologies-A31572) were used for immunofluorescence studies. Human siRNA constructs were purchased from Life Technologies (siPKCζ, siPKCα and siControl catalogue numbers: (10620319-HSS183348, 10620319-HSS183318, 12935112) respectively. TRAF6 siRNA was purchased from Life Technologies, product number s14389-4390824). p38 MAPK inhibitor was purchased from Calbiochem (506126).

4.3.2 Cell Culture and Transfections

A549 and H1299 NSCLC cell lines were maintained in F12K and RPMI-1640 Medium (respectively) supplemented with 10% fetal bovine serum. Cells were kept in a humidified tissue culture incubator at 37°C in 5% CO2. siRNA transfections were conducted using Lipofectamine RNAi max (Life Technologies) according to the manufacturer’s protocol. TGFβ treatments (250 pM) were conducted in serum deprived media (0.2% FBS) for the indicated times after cells were serum deprived overnight.
4.3.3 Protein Concentrations

Protein concentrations were determined using the Lowry method (Fisher).

4.3.4 Immunoblotting and Immunoprecipitation

Cells were lysed (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors) and centrifuged at 14,000 rpm at 4 °C for 10 min. Aliquots of supernatants were collected for analysis of total protein concentration. For immunoprecipitation, equal amounts of remaining cell lysates were incubated with primary antibody, followed by incubation with protein G-Sepharose beads. The precipitates were washed three times with lysis buffer, eluted with sample buffer, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose followed by blocking in 5% skim milk, and incubation with primary antibody in TBST overnight at 4°C. Following incubation with HRP conjugated secondary antibody, proteins were visualized using West Dura Super Signal ECL (Fisher) and imaged on a VersaDoc Imaging system (BioRad).

4.3.5 Cellular Fractionation

Cytoplasmic and Nuclear cellular fractions were isolated using the Thermo Scientific Kit NE-PER® kit (78833) according to the manufacturer’s protocols.

4.3.6 Immunofluorescence Microscopy

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and incubated with primary antibodies at 4°C overnight. Following incubation with the appropriate fluorescent probe conjugated secondary antibodies, the probes were visualized by immunofluorescence microscopy using an inverted IX81 Microscope (Olympus, Canada).
4.3.7 RNA Quality Assessment, Probe Preparation and GeneChip Hybridization

All GeneChips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada; http://www.lrgc.ca). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). Single stranded complementary DNA (sscDNA) was prepared from 200 ng of total RNA as per the Ambion WT Expression Kit for Affymetrix GeneChip Whole Transcript WT Expression Arrays (Applied Biosystems, Carlsbad, CA) and the Affymetrix GeneChip WT Terminal Labeling kit and Hybridization User Manual (Affymetrix, Santa Clara, CA). Total RNA was first converted to cDNA, followed by in vitro transcription to make cRNA. 5.5 ug of single stranded cDNA was synthesized, end labeled and hybridized, for 16 hours at 45°C, to Human Gene 1.0 ST arrays. All liquid handling steps were performed by a GeneChip Fluidics Station 450 and GeneChips were scanned with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA) using Command Console v1.1. Probe level (.CEL file) data was generated using Affymetrix Command Console v1.1. Probes were summarized to gene level data in Partek Genomics Suite v6.5 using the RMA algorithm adjusted for GC content[32]. Using Partek, any batch affect due to scan date was removed and an ANOVA (Yijk = μ + Condition * Timeij+ eijk) using Method of Moments [33] was run to determine gene level p-values. Fold change comparisons are expressed relative to untreated siControl cells, and represent the average of three separate experiments (3 separate gene chips per condition). A fold change of ±1.6 was considered as the cutoff for induction.

4.3.8 Reverse Transcription, Real time PCR and Statistical Analyses

Total RNA was isolated from A549 cells using the RNAeasy Mini kit (Qiagen) according to the manufacturer’s protocol. For complementary DNA (cDNA) synthesis, 1000 ng of total RNA was reverse transcribed using the Superscript® VILO cDNA
synthesis kit according to manufacturer’s protocol (Applied Biosystems). A cDNA equivalent to 10 ng of total RNA was used for all PCR reactions in a total volume of 20 μl. Each reaction was carried out in triplicate. Quantitative PCR (qPCR) reactions were conducted using SsoFast™ EvaGreen® supermix (BioRad) using a Chromo4 Real-time Thermal Cycler (Bio-Rad) according to the recommended protocol of the manufacturer. Primer sequences (5’-3’) are as follows: PKC\(\tau\) (TACGGCCAGGAGATAACC and TCGGAGCTCCAAACAATTC), PKC\(\zeta\) (ATCATTCATGTTTTTCCCGAGCA and GTTGGCAGGTACAGCTTTC), PKC\(\alpha\) (ACAACCTTCAACAACCTTGAC and CCTTCTGTGGCAAGCAT), SNAI-1 (AATCGGAAGCCTAACTACAGCG and GTCCCAGATGAGCATTGGCA), SNAI-2 (ATACCACAACCAGAGATCCTCA and GACTCACTCAGCCCAAGATG), E-cadherin (CCCACCACGTAACACGGGTC and CTGGGTATTTGGGGCATC), MMP9 (CATTTCGACGATGACGAGTTGT and CGGGTGTAGAGTCTCTCGC), PAI-1 (CTCTCTGTGCCCTCAACAC and GTGGAGAGCTCTTGGTCTTG), SMURF2 (GTCCAGAGCTCAGACCGAC and CCAGAGCGGTCTCTCCTTC), TIEG1 (TTCCGGGAACACCTGATTTTC and GCAATGTGAGGTTTGGCAGTA), DAPK1 (AGCTTCGGCTCAAATCCCAAT and TCTCCTTCTCGGTTCTTGATG), Beta-Actin (GGGAATCGTGCCTGACATTAAG and TGTGTTGCGTGACAGGTCTTTC), and POLR2A (GGATGACCTGACTCAACAAACT and CGCCCAGACTCCTTCAGG). Primers were selected using Primer3 [34] as well as PrimerBank [35-37]. Baseline and threshold for Ct calculation were set manually using the Opticon Monitor 3.1 Software (Bio-Rad). PCR efficiencies (E) were calculated using cDNA dilution curves and were > 90% for all genes assessed. Calculated PCR efficiencies were used for gene expression quantification using the Pfaffl formula[38], ratio = \((E_{target})^{\Delta Ct}\text{target(control-treated)} / (E_{reference})^{\Delta Ct}\text{ref(control-treated)})\), where control = siControl, no TGF\(\beta\). Final ratios were calculated using geometric averaging [39] from two reference genes: POLR2A, a gene which was found to be a suitable reference gene in NSCLC models [40], and \(\beta\)-Actin. Gene expression of each treatment is expressed in relation to the control (siControl, no TGF\(\beta\)) and is an average of 3-6 independent experimental trials. Two-way ANOVA analysis followed by post-hoc Bonferonnis’s Tests were used to evaluate the significance of the results.
Statistical analyses were performed using GraphPad Prism® Software 5.0 and p values of <0.05 were considered statistically significant.

4.3.9 Cell Death Assays

A549 and H1299 cells transfected with the appropriate siRNA constructs were serum deprived (0.2% FBS), and then incubated with or without TGFβ in serum deprived media in the presence or absence of a p38 MAPK inhibitor for 48 hours. After 48 hours, apoptosis of A549 and H1299 cells was analyzed by examining nuclear morphology after Hoechst 33342 staining. Hoechst stain (1 ug/mL) was added directly to the medium and incubated for 30 minutes at 37°C. The cells were then visualized using a fluorescent microscope (Olympus IX71), and ten random images were acquired per condition. Normal, and apoptotic nuclei were counted and the apoptotic nuclei (characterized by condensed chromatin) were scored as a proportion of normal/healthy cells.

4.3.10 Statistical Analysis

One-way or Two-way ANOVA analyses followed by post-hoc Bonferonni’s Test were used to evaluate the significance of the results. Statistical analyses were performed using GraphPad Prism® Software 5.0 and p values of <0.05 were considered statistically significant.

4.4 Results

4.4.1 Knockdown of Atypical PKC isoforms alters TGFβ induced gene expression

We have previously reported that aPKC gene silencing using small interfering RNA (siRNA) leads to a temporal extension of TGFβ induced Smad2 phosphorylation [25], and that aPKC facilitates Par6 signalling [26]. Here we assessed global
transcriptional changes in aPKC-silenced cells using gene array (microarray) analysis. Using small interfering RNA (siRNA) targeted to aPKC iota (siPKC\textalpha) and zeta (siPKC\textzeta), we were able to successfully reduce protein levels of both aPKC \textalpha and \textzeta individually, as well as together (siPKC\textalpha/\textzeta) in A549 lung adenocarcinoma cells (Figure 4.1A). As we have previously reported in these cells, knockdown of PKC\textalpha alone results in a compensatory expression of PKC\textzeta (Figure 4.1A). Furthermore, as reported previously, knockdown of aPKC temporally extends P-Smad2 levels 4.5 hours after TGF\beta treatment (Figure 4.1B) as well as 24 hours following a pulse of TGF\beta stimulation (Figure 4.1C) [25].

To conduct the microarray analyses, we stimulated siRNA treated cells (siControl vs. siPKC\textalpha/\textzeta double knockdown) with TGF\beta for 1 hour, followed by washout and further incubation of cells for 24 hours in serum-deprived media. Total RNA was extracted and submitted to the London Regional Genomics Centre for microarray analyses. The raw data were processed and normalized as outlined in the Methods section and a summary table of selected TGF\beta gene changes is presented in Table 4.1.

We summarized fold change differences between siControl and siPKC\textalpha/\textzeta cells after TGF\beta induction (Table 4.1). This list was selected from a set of commonly known genes to be regulated by TGF\beta, modified from [7]. The full microarray data set can be accessed online at the NCBI Gene Expression Omnibus website (GEO; GSE26241). Interestingly, there are several classical TGF\beta genes that show similar expression patterns between control and aPKC silenced cells including BMP4, SNAI1, and SNAI2. However, there were several genes that had muted TGF\beta-dependent gene changes in aPKC-silenced cells compared to control cells, including IL1\alpha, SMURF2, MMP2, and MMP9. We next followed up on these gene changes using real-time PCR.
Figure 4.1
Figure 4.1. aPKC knockdown prolongs TGFβ induced Smad2 phosphorylation

(A) A549 cells transfected with the indicated siRNA were grown for 24 hours and then lysed. Cell lysates were processed for SDS-PAGE and immunoblotted with anti (α)-PKCι, anti-PKCζ, or anti-Actin antibodies as indicated to observe protein knockdown levels.

(B) A549 cells transfected with the indicated siRNA were serum starved and treated with 250 pM TGFβ for 30 minutes, washed, and further incubated for 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with anti (α)-phospho-specific Smad2 or Smad2/3 antibodies. A representative immunoblot from 3 independent replicate experiments is shown.

(C) A549 cells transfected with the indicated siRNA were serum starved and treated with 250 pM TGFβ for 1 hour, washed and further incubated for 24 hours in serum deprived media prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with (α)-phospho-specific Smad2 or Smad2/3 antibodies. A representative immunoblot from 3 independent replicate experiments is shown.
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Table 4.1
Table 4.1. aPKC knockdown alters TGFβ gene response by microarray analysis

A549 cells transfected with control (siControl) or siRNA directed at aPKC isoforms (siPKCι/ζ) were serum starved and treated with 250 pM TGFβ for 1 hour, washed and further incubated for 24 hours in serum deprived media. Total RNA was then extracted and subjected to microarray analysis. Shown is a selected list of genes that differed between control and aPKC silenced cells after 24h for several genes regulated by TGFβ (list adapted from Table 1 in [7]) Fold change comparisons are expressed relative to untreated siControl cells (siControl, -TGFβ), and represent the average of three separate experiments (n=3). In bold are some genes that show a muted response in aPKC knockdown cells.
4.4.2 Knockdown of Atypical PKC alters TGFβ induced gene expression in qPCR analyses

We next re-examined some of the changes we observed in microarray analysis by qPCR. Control and aPKC-silenced cells were treated as described above in the microarray analyses. However, in this case, total RNA was extracted, reverse transcribed, and subjected to qPCR analysis for various TGFβ induced genes. SNAI-1, SNAI-2, E-cadherin, MMP9, PAI-1, Smurf2, DAPK1, and TIEG 1 were tested for relative gene expression in TGFβ induced control or aPKC knockdown cells. We also tested expression of PKCι, PKCζ, and PKCα to confirm aPKC knockdown efficiency. Relative gene expression was normalized using 2 reference genes (Beta-Actin and POLR2A) as described in the Methods section. The graphs represent the average gene expression from at least 3 independent experimental trials.

Figure 4.2A indicates that siRNA targetting aPKC iota and zeta was successful at reducing gene expression of the atypical PKCs (aPKCι and ζ), but not the classical isoform PKCα. Figure 4.2B shows the gene expression of the various TGFβ-induced genes in aPKC knockdown and control cells. SNAI-1 (Snail), SNAI-2 (Slug) and E-cadherin are TGFβ dependent genes involved in the maintenance of epithelial cell junctions. In invasive cancers, TGFβ is known to trigger breakdown of epithelial cell junctions to promote an invasive phenotype by reducing E-cadherin levels through the transcriptional repressors SNAI-1 and SNAI-2 [17]. Our results indicate the transcription factors SNAI-1 and SNAI-2 increase with TGFβ treatment. Furthermore, E-cadherin gene expression is reduced with TGFβ treatment. These results were similar in both control and aPKC silenced cells indicating that aPKC may not be important for transcriptional regulation of these genes. However, several other genes were dampened with aPKC knockdown.

Smurf2 (SMAD ubiquitination regulatory factor 2), is a regulatory factor for the TGFβ receptors, typically involved in targeting Smads and receptors for ubiquitin mediated degradation [8, 13]. Our results indicate that upon TGFβ stimulation, there is an increase in SMURF2 gene expression, however, this effect is abrogated in aPKC-silenced cells.
TGFβ signalling is often involved in inducing expression of genes that are involved in the remodelling of the extracellular matrix and degradation of the basement membrane. These types of TGFβ induced gene changes are implicated in the progression of fibrotic disorders as well as metastatic phenotypes in cancer. Two such genes regulated by TGFβ are PAI-1 (plasminogen activator 1) and MMP9 (matrix metalloproteinase 9)[7, 41]. In observing the gene expression of PAI-1, we saw that TGFβ stimulation induced a significant increase in PAI-1 expression in siControl cells. However, PAI-1 induction was significantly reduced in aPKC silenced cells. Similarly, MMP9 gene expression increases with TGFβ induction and this effect is also significantly reduced in aPKC-silenced cells.

TGFβ Smad signalling can also stimulate pro-apoptotic effects in the epithelium through the positive regulation of genes such as DAPK1 (death-associated protein kinase 1) and TIEG1 (TGFβ-inducible early response gene 1) [19]. Interestingly, we found that in A549 cells, TGFβ actually stimulates a modest decrease in DAPK1 and no change in TIEG1 expression. No significant differences were observed in aPKC-silenced cells compared to control.

Notably, as we observed in the microarray data, the significant differences we observed in TGFβ-induced expression in aPKC knockdown cells were actually decreased gene response. This is despite having temporally extended P-Smad2 levels. We next sought to determine what might be causing this muted TGFβ response.
Figure 4.2
**Figure 4.2. aPKC silencing alters TGFβ dependent gene induction**

Real time PCR analysis of TGFβ induced mRNA levels in A549 control siRNA cells as compared to aPKC double silenced cells (siPKCα/ζ). RNA extracts were isolated from cells treated for one hour with TGFβ followed by 24 hours of incubation in the absence of ligand. Two-way ANOVA analysis followed by post-hoc Bonferroni’s tests were used to determine statistical significance of gene changes (PKCα, PKCζ, PKCζ, CDH1, SNAI1, SNAI2, n=3; all other genes n=6, ±SEM, Two-Way Anova *=p<0.05) a= significant change with TGFβ; b= significant change with aPKC knockdown (A) aPKC siRNA is specific. aPKC knockdown was effective in silencing aPKC gene expression, but not classical PKCα expression. (B) TGFβ effected similar changes in SNAI-1, SNAI-2, E-cadherin, and DAPK1 in control and aPKC silenced cells. However, aPKC knockdown results in reduced TGFβ induced MMP9, PAI-1 and Smurf2 expression. TIEG1 was unaffected by TGFβ treatment or aPKC knockdown.
4.4.3 aPKC knockdown reduces TGFβ induced Smad2 nuclear accumulation

Activated TGFβ receptors phosphorylate receptor regulated Smads (Smad2 and Smad3) on a C-terminal SSXS motif which facilitates their accumulation in the nucleus [8]. Since the reduced transcriptional response we were observing in aPKC silenced cells may be due to a reduced nuclear translocation of Smad2, we examined the cytosolic-nuclear translocation of Smad2 in control and aPKC silenced cells using immunofluorescence microscopy (Fig 4.3A). As expected, in cells transfected with control siRNA, TGFβ induced an increase in Smad2 nuclear staining, indicating a robust nuclear accumulation of Smad2 (Fig 4.3A). However, aPKC silenced cells showed a reduced nuclear accumulation of Smad2 in response to TGFβ. To verify this observation, we also conducted subcellular fractionation studies and immunoblotting of cellular cytosolic and nuclear fractions when cells were treated in the presence or absence of TGFβ. Consistent with our immunofluorescence microscopy analysis, TGFβ treatment stimulates an increase in nuclear Smad2 levels in cells transfected with control siRNA (Figure 4.3B). This is in contrast to aPKC knockdown cells, which show significantly reduced nuclear Smad2 levels upon TGFβ addition. We next assessed whether knockdown of aPKC was mediating effects specific to the TGFβ pathway or whether it was affecting general nuclear import.
Figure 4.3
Figure 4.3. aPKC knockdown reduces TGFβ induced Smad2 nuclear accumulation

(A) A549 cells were transfected with the indicated siRNA, serum starved and treated with 250 pM TGFβ for 1 hour. The cells were processed for immunofluorescence microscopy with antibodies against Smad2. DAPI was used to visualize DNA. Representative images from at least 3 independent replicate experiments are shown. Bar = 10 µm

(B) A549 cells were transfected and treated with TGFβ as described in panel A. The cells were then subjected to subcellular fractionation to isolate cytoplasmic and nuclear fractions. The fractions were subjected to SDS-PAGE and immunoblotted using anti (α)-Smad2, anti-tubulin, and anti-Histone H3 antibodies to determine the subcellular distribution of Smad2. Histone H3 and Tubulin antibodies were used as loading controls for the nuclear and cytoplasmic fractions, respectively. Average nuclear Smad2 levels from 3 independent replicate experiments were quantitated by densitometrical analysis and graphed below the representative immunoblots. (n=3 ± SEM, Two-way ANOVA, *p<0.05.)
4.4.4 siRNA targeting aPKC does not alter TNFα induced NF-κB nuclear translocation

After finding that the cytosolic retention of Smad2 was increased in aPKC silenced cells, we next sought to determine whether aPKC knockdown might be altering general nuclear import. To test this, we examined the classical NF-κB pathway, wherein subsequent to TNFα stimulation, NF-κB translocates to the nucleus via a classical importin dependent mechanism [42]. Using immunofluorescence microscopy we examined the subcellular localization of NF-kB in cells treated with or without TNFα (Figure 4.4). In cells transfected with either control siRNA or siRNA targeting aPKC, NF-κB showed a robust accumulation in the nucleus following TNFα stimulation (Figure 4.4). This suggested that the reduction in Smad2 nuclear localization in aPKC silenced cells was not likely due to an inhibition of general nuclear import machinery and therefore was possibly specific to the Smad pathway. We next sought to determine the cause of cytosolic retention of Smad2 in aPKC silenced cells.
Figure 4.4. aPKC knockdown does not alter TNFα induced NF-κB nuclear accumulation

A549 cells transfected with the indicated siRNA were serum deprived, treated with 10ng/mL TNFα for 30 minutes, and then fixed and processed for immunofluorescence microscopy with antibodies against NF-κB to observe NF-κB subcellular localization. DAPI was used to visualize DNA. Representative images from 3 independent replicate experiments are shown. Bar = 10 µm.
4.4.5 Knockdown of aPKC enhances P-p38 MAPK levels

R-Smads shuttle to and from the nucleus and their subcellular localization is primarily controlled through their C-terminal phosphorylation. The protein structure of Smads has been largely conserved among species and consists of 2 Mad homology domains (MH1 and MH2) connected by an intermediate, proline rich linker region [8]. Interestingly, this linker region contains multiple phosphorylation sites that have been shown to alter Smad localization and function [10]. Originally, linker phosphorylation was discovered to occur through ERK MAPK (via Ras) to exclude Smad from the nucleus [43]. Given our observation of reduced nuclear accumulation of Smads in aPKC silenced cells, we next assessed whether MAPK pathways were altered in aPKC depleted cells. We analyzed the levels of activated MAPK pathways in response to a pulse of TGFβ in control and aPKC silenced cells (Figure 4.5). We noted that at 1 and 24 h time points there did not appear to be a difference between siControl and siPKCα/ζ cells with regards to the levels of phosphorylated ERK 1/2. However, aPKC knockdown increased basal levels of phosphorylated p38 MAPK and extended the duration of p38 phosphorylation to at least 24 hours (Figure 4.5A). Appreciable levels of P-JNK (the last MAPK) were not detected in this cell line (Figure 4.5A). We went on to test a shorter time course of p38 MAPK activation in control and aPKC knockdown cells and discovered that in aPKC-silenced cells (single, or double knockdown) TGFβ-induced p38 MAPK phosphorylation was increased and extended in duration compared to control cells (Figure 4.5B). Because MAPK crosstalk is well known to alter Smad nuclear-cytoplasmic shuttling dynamics [10, 44, 45], we hypothesized that increased p38 MAPK activity may be altering Smad2 nuclear import in aPKC-silenced cells. Thus, after determining that aPKC knockdown was enhancing p38 MAPK signalling, we next sought to test whether this enhanced p38 MAPK activity might play a role in Smad2 nuclear entry.
Figure 4.5
Figure 4.5. aPKC knockdown increases and prolongs p38 MAPK phosphorylation in response to TGFβ

(A) A549 cells transfected with control siRNA (siControl) or siRNA directed at the aPKC isoforms (PKC\(\alpha/\zeta\)) were treated with or without 250 pM TGFβ for 1 or 24 hours prior to lysis. Samples were then processed for SDS-PAGE and immunoblotted with anti (\(\alpha\))-phospho-specific antibodies directed at phosphorylated forms of ERK, p38, and JNK as indicated on the right of the panels. Shown are representative immunoblots from at least 3 independent replicate experiments. Immunoblotting for Actin was used as a loading control.

(B) A549 cells transfected with the indicated siRNA were serum starved and treated with 250 pM TGFβ for 30 minutes, washed, and further incubated for 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with the anti (\(\alpha\))-phospho-specific p38 and total p38 MAPK antibodies as indicated on the right of the panels. Average P-p38 MAPK levels from 3 independent replicate experiments were quantitated by densitometrical analysis and graphed below the representative immunoblots. (n=3 ± SEM, Two-way ANOVA, *p<0.05, **p<0.01).
4.4.6 Inhibition of p38 MAPK does not rescue Smad2 nuclear translocation

Given that we observed increased levels of P-p38 MAPK, we next wanted to assess whether p38 MAPK was responsible for the altered Smad2 nuclear translocation observed in aPKC silenced cells. To carry this out, we pharmacologically inhibited p38 activity in aPKC silenced cells and assayed for Smad2 nuclear translocation by immunofluorescence microscopy. As we previously observed, in cells transfected with control siRNA, TGFβ induced a robust nuclear accumulation of Smad2, whereas in aPKC knockdown cells Smad2 nuclear accumulation was impaired (Figure 4.6). Interestingly, we found that the p38 MAPK inhibitor did not rescue Smad2 nuclear translocation in aPKC silenced cells (Figure 4.6). This result suggested that p38 MAPK activity was likely not responsible for the reduced Smad2 nuclear accumulation observed in aPKC silenced cells. Although, we found that inhibiting p38 MAPK did not rescue Smad2 nuclear accumulation, we wanted to address whether the enhanced p38 MAPK levels we observed might be altering TGFβ induced apoptosis.
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**Figure 4.6**
Figure 4.6. p38 MAPK inhibition of aPKC knockdown cells does not rescue Smad2 nuclear accumulation

A549 cells transfected with the indicated siRNA, were serum deprived in the presence or absence of a p38 MAPK inhibitor, and then treated with 250 pM TGFβ for 1 hour. Cells were then fixed and processed for immunofluorescence microscopy with antibodies against Smad2 to observe Smad2 subcellular localization. DAPI was used to visualize DNA. Representative images from at least 3 independent replicate images are shown. Bar = 10 µm.
4.4.7 Knockdown of aPKC increases TGFβ induced apoptotic response via p38 MAPK

TGFβ receptors can activate the p38 MAPK pathway to stimulate apoptosis [18, 46]. We next examined whether the increased p38 MAPK signalling observed in aPKC silenced cells could sensitize cells to TGFβ induced apoptotic response. To measure apoptosis, control and aPKC silenced cells were treated with or without TGFβ for 48 hours, and the percentage of apoptotic cells was measured through the assessment of nuclear morphology after Hoescht staining. Cells treated with control SiRNA showed a modest apoptotic response to TGFβ, which is in contrast to aPKC-silenced cells, which exhibited a significant increase in cell death when treated with TGFβ (Figure 4.7A). Importantly, treatment of aPKC silenced cells with a p38 MAPK inhibitor reduced the number of apoptotic nuclei, indicating the apoptotic response observed was downstream of p38 MAPK. Furthermore, cleaved-Parp levels (a marker of apoptosis) induced by TGFβ were significantly higher in aPKC silenced cells than in control cells (Figure 4.7B). Similar to the above results, p38 MAPK inhibition reduced cleaved-Parp levels in aPKC silenced cells indicating that the apoptotic response observed in aPKC silenced cells likely involved the p38 MAPK pathway (Figure 4.7B). We next tested another NSCLC cell line, H1299 cells, for apoptotic response and Smad2 nuclear accumulation to assess whether the results we were observing were cell type specific.

4.4.8 Knockdown of aPKC also mediates TGFβ effects in H1299 NSCLC cells

We next examined whether the results we observed in A549 cells could be recapitulated in H1229 cells, a second NSCLC cell line. We tested whether aPKC knockdown in H1299 cells altered Smad2 nuclear accumulation upon TGFβ stimulation. Using an immunofluorescence approach as we have done above (Figure 4.3A), we examined the localization of Smad2 after TGFβ treatment in control, and aPKC knockdown cells. Similar to our findings with A549 cells, TGFβ treatment increased the nuclear localization of Smad2 in H1299 cells transfected with control siRNA (Figure
4.8A). Furthermore, siRNA targeting aPKC reduced TGFβ induced nuclear accumulation of Smad2 (Figure 4.8A). We also tested whether aPKC knockdown cells showed enhanced p38 MAPK phosphorylation by treating cells with TGFβ in a timecourse as we have done before for A549 cells. As we had seen in A549 cells, aPKC-silenced H1299 cells also showed an enhanced p38 MAPK response compared to control cells (Figure 4.8B). Furthermore, we also observed that this increased p38 MAPK signalling correlated with an increased apoptotic response in aPKC knockdown cells (Figure 4.8C), and that this effect was abrogated with a p38 MAPK inhibitor. Importantly, these results are in agreement with our A549 cell data. After seeing that the results we had observed did not seem to be cell type specific, we next wanted to investigate the mechanisms for these altered responses in aPKC knockdown cells. The TGFβ signalling pathway to p38 MAPK involves the recruitment and activation of the ubiquitin ligase TRAF6 [21, 22]. To gain mechanistic insight into why aPKC silenced cells showed enhanced p38 MAPK levels, we next examined the role of TRAF6 in this pathway.
Figure 4.7
Figure 4.7. aPKC knockdown enhances TGFβ induced apoptotic response

(A) A549 cells transfected with control siRNA (siControl) or siRNA directed at the aPKC isoforms (PKCθ/ζ) were serum deprived and treated with or without 250pM TGFβ for 48 hours in the presence or absence of a p38 MAPK inhibitor. Hoescht 33342 was used to stain the nuclei of cells prior to image acquisition and cell counting. Quantification of apoptotic nuclei (yellow arrowheads) from four independent experiments are expressed graphically to the right of the representative images. (n=4±SEM, Two-way ANOVA, *=p<0.05)

(B) A549 cells were treated as in (A) and then lysed. Cell lysates were processed for SDS-PAGE and immunoblotted with anti (α)-cleaved PARP and anti-Actin antibodies. Average densitometrical analysis from 4 independent replicate experiments is shown graphically below the representative immunoblot. (n=4±SEM, Two-way ANOVA, *=p<0.05.)
Figure 4.8
Figure 4.8. aPKC knockdown in H1299 cells also reduces Smad2 nuclear accumulation, increases p38 MAPK levels, and enhances apoptosis, similar to A549 cells

(A) H1299 NSCLC cells were transfected with the indicated siRNA, serum starved and treated with 250 pM TGFβ for 1 hour. The cells were processed for immunofluorescence microscopy with antibodies against Smad2. DAPI was used to visualize DNA. Representative images from at least 3 independent replicate experiments are shown. Bar = 10 µm

(B) H1299 NSCLC cells transfected with the indicated siRNA were serum starved and treated with 250 pM TGFβ for 30 minutes, washed, and further incubated for 1 or 4 hours prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with anti-(α)-phospho-specific p38 and total p38 MAPK antibodies as well as anti-PKCδ and anti-PKCζ antibodies as indicated on the right of the panels. Representative immunoblots from at least 3 independent replicate experiments is shown.

(C) H1299 NSCLC cells transfected with control siRNA (siControl) or siRNA directed at the aPKC isoforms (PKCδ/ζ) were serum deprived and treated with or without 250pM TGFβ for 48 hours in the presence or absence of a p38 MAPK inhibitor. Hoescht 33342 was used to stain the nuclei of cells prior to image acquisition and cell counting. Quantification of apoptotic nuclei (yellow arrowheads) from four independent replicate experiments are expressed graphically to the right of the representative images. (n=4±SEM, Two-way ANOVA, **= p<0.01)
4.4.9 Knockdown of aPKC increases TβRI-TRAF6 complexes

TGFβ-stimulated apoptosis mediated via p38 MAPK has been previously reported to occur through the recruitment and activation of the E3 ubiquitin ligase TRAF6 [21, 22]. Briefly, upon TGFβ activation, TRAF6 is recruited to TβRI of the TGFβ receptor complex. This causes TRAF6 to become auto-ubiquitinated, which activates TAK1 (a MAP3K), which in turn triggers the MAPK cascade to p38 activation [21, 22]. We have previously shown that aPKC expression can alter binding patterns of TβRI substrates [26]. We hypothesized that the increased TGFβ-p38 MAPK signals we observed in aPKC silenced cells, may have been due to an increased association of TRAF6 with TGFβ receptors when aPKC was depleted. Indeed we have also previously shown that overexpressed aPKC can negatively regulate steady-state TGFβ receptor levels and that knockdown of aPKC reduces the rate of activated TGFβ receptor complex degradation [25]. We tested this idea by immunoprecipitating endogenous TRAF6 from control, and aPKC silenced cells, followed by immunoblotting for exogenously expressed TβRI (Figure 4.9). Interestingly, TβRI associated to a greater degree with TRAF6 in the absence of aPKC expression (Figure 4.9). This finding suggested that TβRI-TRAF6 complexes were more stable in aPKC knockdown cells. We reasoned that this increase in TβRI-TRAF6 complexes may have led to increased TGFβ-induced p38 MAPK signals in aPKC silenced cells. To test this idea, we next examined whether TRAF6 knockdown could abrogate the p38 MAPK signalling seen in aPKC silenced cells.
Figure 4.9. aPKC knockdown increases levels of TβRI-TRAF6 complexes

HEK 293T cells transfected with control siRNA (siControl) or siRNA targeting aPKC isoforms (PKC\(\alpha/\zeta\)) were co-transfected with cDNA encoding Flag-tagged TGF\(\beta\) type 1 receptor (FlagTβRI) as indicated. Cells were then lysed and endogenous TRAF6 was immunoprecipitated (IP) using anti-TRAF6 antibodies. The immunoprecipitates were processed for SDS-PAGE and immunoblotted with anti-(\(\alpha\))-Flag and anti-TRAF6 antibodies to visualize immunoprecipitated Flag-tagged TβRI and TRAF6 (top panel). Cell lysates were immunoblotted with anti- PKC\(\alpha\), anti-PKC\(\zeta\), anti-Flag and anti-TRAF6 antibodies to visualize endogenous aPKC and TRAF6 levels as well as expressed Flag-tagged TβRI (bottom panel). Representative immunoblots from at least from 3 independent replicate experiments are shown.
4.4.10 Knockdown of TRAF6 in aPKC silenced cells reduces activated p38 MAPK and apoptosis

Because aPKC knockdown was enhancing P-p38 MAPK levels in response to TGFβ, we inferred that knockdown of TRAF6 may reduce this effect. We used siRNA to knockdown the aPKCs alone, TRAF6 alone, or aPKC and TRAF6 together. As we had seen before, aPKC silencing increased P-p38 MAPK levels in response to TGFβ (Figure 4.10A). Interestingly, in cells where we depleted protein levels of aPKC and TRAF6 simultaneously by siRNA knockdown, TGFβ-induced p38 MAPK phosphorylation was abrogated (Figure 4.10 A). Furthermore, as we had observed before, aPKC knockdown cells exhibited an increased TGFβ-induced apoptotic response as measured by Hoescht staining and quantitation of pyknotic nucleic (Figure 4.10B). In contrast, simultaneous knockdown of TRAF6 and aPKC reduced the TGFβ-induced apoptotic effect (Figure 4.10B), which corresponds to the reduced p38 MAPK phosphorylation seen in these cells (Figure 4.10A). These results suggested that the enhanced p38 MAPK signalling and apoptosis we had observed in aPKC silenced cells were TRAF6 dependent. Although, we found that p38 MAPK was enhancing apoptosis via a TRAF6 dependent mechanism in aPKC depleted cells, we still did not resolve why Smad2 nuclear translocation was reduced. We therefore next assessed Smad2-Smad4 complex formation in aPKC knockdown cells.
Figure 4.10
Figure 4.10. Knockdown of TRAF6 abrogates TGFβ-p38 MAPK effects observed in aPKC depleted cells

(A) A549 cells transfected with control siRNA, or siRNA directed at aPKC (siPKCα/ζ), TRAF6 (siTRAF6), or both aPKC and TRAF6 (siPKCα/ζ + TRAF6) were serum starved and treated with 250 pM TGFβ for 1 hour prior to lysis. Lysates were then processed for SDS-PAGE and immunoblotted with the anti (α)-phospho-specific p38 and total p38 MAPK antibodies as indicated on the right of the panels. Immunoblotting using anti-TRAF6, anti-PKCα, and anti-PKCζ antibodies were used to determine knockdown levels. Average P-p38 MAPK levels from 3 independent replicate experiments were quantitated by densitometrical analysis and graphed below the representative immunoblots (n=3±SEM, Two-way ANOVA, **= p<0.01).

(B) A549 cells transfected as in (A) were serum deprived and treated with or without 250 pM TGFβ for 48 hours. Hoescht 33342 was then used to stain the nuclei of cells prior to image acquisition and cell counting. Quantification of apoptotic nuclei (yellow arrowheads) from three independent experiments are expressed graphically below the representative images. (n=3±SEM, Two-way ANOVA, *=p<0.05).
4.4.11 Knockdown of aPKC reduces Smad2-Smad4 complex formation

In the basal state, Smad2 is predominantly localized to the cytoplasm, however, in response to phosphorylation, Smad2 associates with Smad4, and accumulates in the nucleus [2, 8]. Although phosphorylated R-Smads can activate transcription alone, a full TGFβ response requires complex formation of Smad2 and Smad4 [47]. We next examined whether aPKC silencing was altering TGFβ-induced Smad2-Smad4 interaction by immunoprecipitating Smad2 and immunoblotting for Smad4 in Control and aPKC silenced cells (Figure 4.11). As expected, in cells transfected with control siRNA, the addition of TGFβ increased the amount of Smad4 co-immunoprecipitating with Smad2 indicating that TGFβ treatment induced Smad2-Smad4 complex formation (Figure 4.11). In contrast, aPKC silenced cells exhibited a significantly reduced level of Smad2 associated Smad4 with TGFβ addition (Figure 4.11). This indicated that the reduction in gene response we observed in aPKC silenced cells may be due to reduced Smad2-Smad4 interaction. We next examined whether SARA could be playing a role in this process.
Figure 4.11
Figure 4.11. aPKC knockdown reduces TGFβ-induced Smad2-Smad4 complex formation

A549 cells transfected with control siRNA (siControl) or siRNA directed at the aPKC isoforms (PKCθ/ζ) were serum starved and treated with or without 250 pM TGFβ for 1 hour prior to lysis. Cell lysates were then immunoprecipitated (IP) using anti (α)-Smad2 antibodies, followed by immunoblotting for Smad4 to determine the level of induction of Smad2-Smad4 complex formation. Cell lysates were included to show relative endogenous protein expression. Average densitometrical analysis from three independent replicate experiments is shown below the representative immunoblots. (n=3 ±SEM, Two-way ANOVA, **=p<0.01).
4.4.12 Knockdown of aPKC increases SARA expression

The subcellular localization of R-Smads can be controlled by a balance between binding factors that retain them in the cytoplasm vs. transcription factors that retain them in the nucleus. One such cytoplasmic retention factor is the Smad anchor for receptor activation (SARA), an early endosome anchored FYVE domain containing protein. Under normal conditions, Smad2 can be anchored to the early endosome by SARA [12, 15, 48]. Originally the function of SARA was described to recruit non-phosphorylated Smad2 to the activated receptor complex [12, 15, 48]. However, a more complex role for SARA is emerging, as recent reports have indicated that SARA may be dispensable for TGFβ signalling [49], and also that SARA may be involved in more general endocytic trafficking mechanisms [50]. Because we have previously reported alterations in membrane trafficking of TGFβ receptors upon PKC inhibition ([25]; Chapter 2), we next examined whether SARA levels were altered in aPKC silenced cells. Interestingly, aPKC silenced cells showed an increased total protein expression of SARA compared to control cells (Figure 4.12A), although no appreciable alterations were observed in early endosome localization with aPKC knockdown by immunofluorescence microscopy (Figure 4.12B). We next analyzed whether the increased SARA levels could be retaining Smad2 in the cytoplasm.

4.4.13 Knockdown of aPKC increases cytosolic retention of Smad2 by SARA

SARA preferentially binds unphosphorylated forms of Smad2, and it is thought that the activated receptor complex formed at the plasma membrane is captured by SARA in the early endosome, which then presents the bound R-Smad to the receptor for phosphorylation [2]. Smad2 then dissociates from SARA and associates with Smad4 prior to nuclear translocation and the initiation of transcription [2]. We examined whether the increased SARA levels in aPKC silenced cells correlated with enhanced cytoplasmic retention. We immunoprecipitated Smad2 from control and aPKC silenced cells treated with TGFβ to examine whether SARA was dissociating from Smad2 upon TGFβ
addition. As expected, TGFβ addition causes a dissociation of SARA from Smad2 (Figure 4.13). Smad2 dissociation from SARA is followed by a concomitant increase in the binding of Smad4, similar to what we observed in Figure 4.11. Interestingly, in aPKC silenced cells TGFβ addition reduced SARA-Smad2 dissociation, and also reduced the binding of Smad2 to Smad4 (Figure 4.13). This implied that SARA was retaining Smad2 in the cytoplasm to a greater degree in aPKC silenced cells, and was likely responsible for the observed reduced nuclear translocation of Smad2 that we had observed. Thus, in summary, we found that aPKC silenced cells showed reduced Smad2 nuclear accumulation in response to TGFβ probably due to an increased cytoplasmic retention by SARA, and furthermore, aPKC silenced cells showed increased TβRI-TRAF6 complex levels, and enhanced TGFβ-induced p38 MAPK phosphorylation and apoptotic response (summarized in Figure 4.14).
Figure 4.12
Figure 4.12. Knockdown of aPKC increases steady state SARA protein levels

(A) A549 cells transfected with control siRNA (siControl) or siRNA targeting aPKC isoforms (PKCθ/ζ) were lysed and immunoblotted using antibodies for SARA and Actin as indicated on the right of the panels. Average densitometrical analysis of steady state SARA levels from three independent experiments is shown graphically to the right of the representative immunoblots. (n=3±SEM, Two way ANOVA, *=p<0.05).

(B) aPKC knockdown does not inhibit the localization of SARA in the early endosome. A549 cells were transfected as described in Panel A and processed for immunofluorescence microscopy to visualize EEA1 (green) and SARA (red). DAPI was used to visualize DNA (blue). Representative images from at least 3 independent experiments are shown. Bar = 10μm.
Figure 4.13
Figure 4.13. Knockdown of aPKC reduces TGFβ induced Smad2 release from SARA

A549 cells transfected with control siRNA (siControl) or siRNA directed at the aPKC isoforms (PKCθ/ζ) were serum starved and treated with or without 250 pM TGFβ for 1 hour prior to lysis. Cell lysates were then immunoprecipitated (IP) using anti (α)-Smad2 antibodies, and subjected to SDS-PAGE and immunoblotting using α-SARA, α-Smad4 and α-Smad2 antibodies. IgG heavy chain is indicated. Cell lysates were included to show relative endogenous protein expression. Average densitometrical analysis of Smad2 associated SARA levels from three independent replicate experiments is shown graphically below the representative immunoblots. (n=3 ±SEM, Two Way Anova, *p<0.05).
Knockdown of aPKC increased steady state levels of SARA. aPKC knockdown cells also exhibit reduced Smad2 nuclear accumulation in response to TGFβ due to increased SARA mediated Smad2 cytosolic retention, and reduced Smad2-Smad4 complex formation. Knockdown of aPKC also increased levels of TβRI-TRAF6 complexes, and enhanced TGFβ-induced, TRAF6-dependent p38 MAPK phosphorylation to stimulate apoptotic response.
4.5 Discussion

The TGFβ pathway controls an array of developmental and homeostatic processes and alterations in the pathway are associated with various pathologies such as fibrosis and cancer. Given the varied responses associated with a TGFβ signal, it is clear that the context dependent execution of signalling may be regulated at multiple levels. We have recently demonstrated an important role for aPKC in regulating TGFβ signals. More specifically, we have previously reported that aPKC alters TGFβ receptor trafficking, as well as the execution of a full EMT response through Par6 signalling. Here we have found that aPKC knockdown alters both Smad-dependent and the Smad-independent p38 MAPK signalling pathways.

In this report we examine the transcriptional changes associated with TGFβ signalling in an aPKC silenced background. In this context, we found that several TGFβ stimulated genes showed reduced transcriptional activity. We discovered that aPKC silencing played an important role in allowing a full Smad2 nuclear translocation. Interestingly, we found aPKC expression was also required for a full Smad2-Smad4 interaction following TGFβ stimulation. How aPKC controls Smad2-Smad4 dynamics is an area that requires further examination.

One possibility is an alteration in the access of Smad4 for Smad2. The correct subcellular localization of Smad2 is controlled by trafficking of Smads and their association with SARA. In this report we find that the knockdown of aPKC increased the basal protein levels of SARA. This finding is important, because increased SARA levels have been reported to reduce TGFβ receptor degradation, and also to correlate with the maintenance of epithelial phenotype [13, 51]. Given our previous finding that aPKC alters the membrane trafficking of the TGFβ receptors [25], it would be interesting to explore whether aPKC alters the function or localization of SARA to control the context under which Smads are signalling. Although we did not detect appreciable changes in early endosome localized SARA levels, it is possible that in aPKC-silenced cells, the normal trafficking of SARA (and its binding partners) to other subcellular compartments could be altered. Indeed, a very recent report has implicated SARA in general endocytic processes via classical ESCRT complex machinery [50]. More specifically, the correct
subcellular trafficking of the EGFR from the early endosome to late endosomes to regulate EGFR degradation was dependent on SARA, implicating SARA with a more general role in endocytic trafficking than was previously appreciated [50]. This may have important implications with respect to our findings that aPKC knockdown reduces TGFβ receptor degradation and stabilizes particular TGFβ receptor-protein complexes [25, 26]. One possibility is that aPKC controls the normal degradative trafficking of the TGFβ receptors, and depletion of aPKC leads to an accumulation of SARA and TGFβ receptor complexes. Indeed, aPKCs has previously been reported to be involved in the trafficking of membrane proteins, as well as being involved in the passage of EGFR to lysosome targeted endosomes through the anchoring protein p62 [52]. Whether the knockdown of aPKC in our model is causing a reduced passage of receptors to lysosomes is an important area for future study. Furthermore, if this is the case, it would be important to examine whether altered SARA trafficking changes the subcellular availability of Smad4 for Smad2. Interestingly, although TGFβ receptors can phosphorylate Smad2 in the absence of SARA, it has been reported that SARA is required for proper Smad2 nuclear translocation [15]. Furthermore, although inhibiting TGFβ receptor internalization from the membrane only slightly altered phosphorylated Smad2 levels, it did significantly impact the ability of Smad2 to dissociate from SARA [15]. This suggests that the coordinated function and subcellular localization of SARA and associated Smads are important for mediating TGFβ dependent transcription properly.

Interestingly, our transcriptional analyses revealed that in aPKC silenced cells, some genes showed a muted response to TGFβ (e.g. PAI-1, Smurf2, MMP9) but others responded to the same degree (e.g. SNAI1, SNAI2, E-Cadherin). Surprisingly, this is consistent with a report that TGFβ can stimulate two classes of genes: genes which are Smad4 dependent and genes which are Smad4 independent [53]. Using genetic knockdown and microarray analyses, the authors identify PAI-1 and Smurf1 as Smad4 dependent genes, and SNAI-2 as a Smad4 independent gene [53]. This suggests that possibly some of the reduced transcripts we observe in aPKC silenced cells are due to Smad4 not accessing the nucleus in response to TGFβ. This is consistent with our
findings that the SARA-Smad2-Smad4 exchange and Smad nuclear translocation is altered in aPKC silenced cells.

Interestingly, a recent analysis of Smad mutations in colorectal cancer indicates that a great proportion of mutations in Smad4 map to the conserved R-Smad binding surface [54]. Furthermore, the authors report several other R-Smad mutations that reduce Smad4 binding [54] – indicating that loss of Smad complex formation is an important event during colorectal tumor formation. This opens the possibility that a post-translational modification of Smad4 may inhibit Smad2 interaction in aPKC silenced cells. Certainly, this has been shown before, as Smad4 ubiquitination and deubiquitination play pivotal roles in Smad complex formation and disassembly [47, 55]. Interestingly, previous reports have described Smad4 dependent and Smad4 independent gene changes with respect to TGFβ. Whether ubiquitination status of Smad4 alters Smad2 binding in aPKC silenced cells remains to be elucidated.

Here, we also made a novel finding that the knockdown of aPKC increased and prolonged TGFβ-induced p38 MAPK activation, and this sensitized NSCLC cells to apoptosis. We found that knockdown of aPKC stabilized TβRI-TRAF6 complexes, and that knockdown of TRAF6 in aPKC silenced cells returned p38 MAPK activation levels back to control levels. This result is important, because it suggests that the variability seen in p38 MAPK activation by TGFβ in various cell models may be due to factors that destabilize TRAF6-receptor complexes, such as the expression of aPKC.

In line with our results, increases in p38 MAPK activity have been reported before upon aPKC silencing [31] indicating that aPKC may attenuate p38 MAPK signalling in multiple cancer cell types. Interestingly, when aPKC is knocked down, p38 MAPK is able to signal an apoptotic response indicating that in some situations aPKC may be a viable therapeutic target. However, the role of p38 MAPK in cancer is also complex, and context dependent – and in addition to sensitizing cells to a death response, p38 activity is also associated with cancer cell survival and both the stimulation and suppression of EMT [20, 56, 57]. The role of Smad2 linker phosphorylation by MAPK members in TGFβ signalling has also yielded mixed results. The original reports show that linker phosphorylation by MAPK blocked Smad2 nuclear accumulation [43, 58], however, nuclear stabilization of Smad2 by linker phosphorylation has also been reported
suggesting that Smad linker phosphorylation is more complex than originally thought, and thus requires further examination. We report here that the enhanced p38 MAPK activation in aPKC silenced cells was not responsible for the reduction in Smad2 nuclear accumulation. It would be interesting to see whether linker phosphorylation alters Smad2-Smad4 complex formation. In any case, the role of Smad linker phosphorylation is complex and requires further detailed examination.

In conclusion, we have found aPKC plays multiple roles in TGFβ signalling and the localization and expression patterns of aPKC may dictate how a cell responds to TGFβ. This is especially important since aPKCs have recently been implicated cancer progression [28, 60] and aPKCt has been classified as a human oncogene [29, 61]. Although many interesting questions still remain to be answered, our work suggests that aPKCs may alter the way cells respond to TGFβ signals.

4.6 Footnotes

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4.7 References


Chapter 5

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5 Chapter 5

5.1 General Summary

TGFβ regulates pleiotropic signalling pathways that execute key signalling programs, which have important roles in development, tissue homeostasis and disease. It is now clear that the complex and versatile responses to TGFβ signalling are substantially defined by regulatory mechanisms that control the intensity and duration of TGFβ signals, but also by interacting protein partners that can define which particular signal will be propagated. It is also clear that this complex regulation of TGFβ signals is inherently linked to trafficking, as the internalization and subcellular itinerary of the TGFβ receptors can control signalling outcome.

It has been shown that TGFβ signalling through the Smad family of proteins is propagated when the receptors internalize via clathrin-coated pits into the early endosome, whereas TGFβ receptors are more likely to be degraded when they internalize via membrane rafts. Thus, receptor trafficking is an important aspect of whether a Smad signal is propagated or degraded [1]. Furthermore, upon TGFβ stimulation, non-Smad pathways are also initiated. These include the TGFβ-Par6 pathway which stimulates cells to undergo EMT [2, 3], as well as various MAPK pathways including p38 MAPK signalling which can stimulate epithelial cells to undergo apoptosis [4-6]. Understanding the factors that contribute to the regulation of the trafficking and signalling of the TGFβ pathway are an important area of research.

In this thesis I examined the role of the aPKC class of proteins in regulating TGFβ signalling processes and outcomes. aPKC has known roles in endocytic trafficking, thereby linking it to classical TGFβ-Smad signal transduction. Furthermore, aPKC is a direct binding partner of both Par6 and TRAF6 thereby linking it to both the TGFβ-Par6 pathway as well as the TGFβ-p38 MAPK pathway (respectively); however, the role of aPKC in these pathways was not fully understood. The overall purpose of this study was to examine whether aPKC modulates various TGFβ pathways, while characterizing mechanisms and outcomes contributing to these effects. I evaluated whether broad pharmacological PKC inhibition altered membrane trafficking patterns of
the TGFβ receptors, and subsequently whether siRNA knockdown of aPKC isoforms altered TGFβ receptor dynamics and signalling patterns. Furthermore, I examined the role of aPKC in the TGFβ-Par6 pathway, and discovered that both aPKC isoforms could phosphorylate Par6 to facilitate the EMT of NSCLC cells. Finally, I examined the role of aPKC in the initiation of TGFβ genetic program and discovered that aPKC knockdown reduces Smad2 nuclear accumulation through an increased level of SARA mediated retention. Furthermore, I also showed that knockdown of aPKC increases TβRI-TRAF6 complexes and enhances p38 MAPK activation upon TGFβ stimulation leading to increased apoptosis. Thus, I have shown that aPKC is intricately linked to TGFβ signalling, and its expression can modulate TGFβ signalling outcomes. A brief summary of the major findings from this thesis is described in Figure 5.1.
In this thesis I have found that aPKC isoforms affect both Smad-dependent and Smad-independent TGFβ signalling pathways. aPKC plays a role in TGFβ receptor early endosome transit, as well as SARA-Smad2-Smad4 binding dynamics. aPKC also phosphorylates Par6 to facilitate TGFβ induced EMT. Finally, aPKC expression regulates TβRI-TRAF6 complexes to control the ability of TGFβ to signal to p38 MAPK.
5.2 General Discussion and Future Directions

5.2.1 Regulation of TGFβ receptor trafficking and signalling by atypical protein kinase C

Although it is now well established that the endocytic itinerary of the TGFβ receptors can influence TGFβ signalling patterns, the proteins that control these processes are less understood. PKC family members are known regulators of intracellular traffic [7] but their potential role in TGFβ signalling was not explored. In chapter 2 of my thesis I examined the role of PKC in TGFβ signalling by examining the trafficking and degradation patterns of the TGFβ receptors with pharmacological inhibition of PKC. We discovered that broad inhibition of PKC isoforms shifted the TGFβ receptors into the early endosome, and reduced TGFβ receptor degradation. Interestingly, we also pinpointed that the atypical class of PKCs (aPKCs) was responsible for enhancing phospho-Smad2 levels. This is an important finding, because specifying PKC isoforms relevant to particular pathways will aid in the development of selective therapeutic approaches. Interestingly, when we knocked down aPKC using siRNA, we were able to extend TGFβ induced P-Smad2 levels, which corresponded to a reduced TGFβ receptor degradation. However, in chapter 4, when we examined the transcriptional output of this enhanced Smad2 phosphorylation we determined that aPKC knockdown actually reduced TGFβ mediated transcription of several genes. Interestingly, we then found that TGFβ-induced Smad2 nuclear accumulation was reduced in aPKC depleted cells, and we showed this was due to an enhanced capacity of SARA to retain Smad2 in the cytosplasm. This is an interesting finding for a number of reasons. Firstly, it suggests that the proper exchange of SARA-Smad2 complexes to Smad2-Smad4 complexes is a regulated process that may require more than just phosphorylation of the R-Smad. Certainly, it is in line with a previous report that the proper subcellular localization through internalization of the receptors is required for SARA to dissociate from Smad2 upon phosphorylation [8]. This is particularly important, as there has been some controversy with respect to SARA, as some reports indicate SARA as being dispensable for the phosphorylation of R-Smads [9, 10]. Consistent with the above reports, we show that Smad2 still becomes phosphorylated in aPKC knockdown cells, however,
dissociation of Smad2 from SARA is severely impaired, which in turn impairs Smad2 nuclear translocation and transcriptional response. However, many questions still need to be answered. For example, how aPKC knockdown impair SARA-Smad2 dissociation still needs to be identified. One possibility is a change in trafficking dynamics of the TGFβ receptors and SARA. We have shown that the knockdown of aPKC increases the total protein levels of SARA (in chapter 4) and also slows TGFβ receptor degradation (in chapter 2). One possibility is that TGFβ receptor-SARA complexes are being internalized slower in aPKC knockdown cells, and thus are accessing intracellular degradatory machinery (such as Smurf1, Smurf2 or other ubiquitin ligases) at a slower rate. This could effectively explain why we see increased receptor levels, SARA levels, and reduced SARA-Smad2 dissociation, as internalization is required for the degradation of these proteins as well as proper Smad2 dissociation and nuclear translocation [1, 8]. Furthermore, if this is the case, understanding the mechanisms that control the reduced degradation patterns and altered subcellular itinerary of the TGFβ receptors is also needed. Certainly, a recent report implicates SARA in controlling the trafficking patterns of the EGFR through the recruitment of the ubiquitination factor RNF11 [11]. Importantly, aPKC is a known binding partner of the TGFβ pathway ubiquitin ligase Smurf1, and has been shown to recruit Smurf1 to degrade substrate proteins such as RhoA [12]. It would be interesting to test whether aPKC was facilitating the recruitment of Smurf1 to the TGFβ receptor-SARA complex to control their correct trafficking and degradation. If this was the case, presumably in aPKC knockdown cells a lack of Smurf1 recruitment could lead to an accumulation of both TGFβ receptors and SARA, as well as mislocalized intracellular targeting of the activated complex. Thus, whether the ubiquitination status of TGFβ receptor complexes is altered to change subcellular trafficking is an area that requires further investigation. Also, we have not examined whether aPKC controls these dynamics through phosphorylation of the TGFβ receptors. Interestingly, aPKC can phosphorylate the EGFR to change its trafficking itinerary from degradation to recycling [13]. Whether aPKC can phosphorylate TGFβ receptor complexes is an area that needs to be addressed. Another finding we made in chapter 4 was that aPKC knockdown increased TGFβ-receptor-TRAF6 complexes which enhanced
p38 MAPK signalling and subsequent apoptotic response. Interestingly, although it is well accepted that TGFβ can activate MAPK pathways, there has been skepticism about the physiological importance of TGFβ-p38 MAPK signalling, particularly because the activation does not occur in all cell lines, and many times the activation is much lower than classical activators of the p38 MAPK pathway. My data certainly suggests that enhanced TGFβ-TRAF6 receptor complex formation stimulates TGFβ-p38 MAPK signalling; and importantly, this can be controlled by the expression of other proteins, such as aPKC. This suggests that the variability seen in TGFβ-p38 MAPK activation in various cell lines and models may be due to differences in the endogenous levels of TGFβ receptor-TRAF6 complexes. How aPKC knockdown is stabilizing TβRI-TRAF6 is a question that still remains to be answered. Interestingly, some of the ideas mentioned above for the SARA-Smad2 pathway, may also hold here. aPKC may be involved in recruiting a ubiquitin ligase (such as Smurf1) for the degradation of TβRI-TRAF6 complexes upon internalization. Thus, in aPKC knockdown cells, TβRI-TRAF6 is degraded less, and thus can activate p38 MAPK to a greater degree. Again, understanding whether ubiquitination, or aPKC phosphorylation is involved in this process would be interesting areas of study.

Although I have identified aPKC as an important player in signalling patterns of the TGFβ receptors, clearly, a more thorough analysis of the internalization patterns of the TGFβ receptors, and the mechanisms that control them is warranted for understanding both Smad and non-Smad signalling pathways.

5.2.2 Atypical Protein kinase C phosphorylates Par6 to facilitate EMT

EMT is an important step in tumour progression, as it signifies that the once immobile mass of growing carcinoma cells have escaped the physical constraints of the epithelium and are gaining the capacity for independent movement. The TGFβ-Par6 axis was previously shown to be an important pathway in EMT through TβRII mediated phosphorylation of Par6 [2, 3]. In chapter 3, I showed that both aPKC isoforms (α and ζ) phosphorylate Par6 on S345 to stimulate EMT [14]. Importantly, this was the first time
another protein other than TβRII was shown to phosphorylate Par6 on this critical residue. Interestingly, I found that aPKC co-localizes with TGFβ receptors at the leading edge of migrating cells, and that aPKC1 associates with TGFβ receptors through Par6. Furthermore, upon TGFβ stimulation, which triggers EMT in A549 adenocarcinoma cells, aPKC becomes activated as indicated by an increase in its phosphorylation status. We found that both aPKC isoforms are able to phosphorylate Par6, and that both the association with Par6 and kinase activity of aPKC were important for this phosphorylation event. When we silenced aPKC using siRNA we significantly reduced the ability of TGFβ to target RhoA and E-cadherin for degradation and subsequently actin stress fibre formation, EMT and migration were reduced (Figure 5.2A). When we re-introduced a phospho-mimetic Par6 into aPKC silenced cells, we rescued EMT as measured by a restored loss in RhoA and E-cadherin [14].

Given the recent important roles reported for both Par6 and aPKC in the generation and progression of various cancers, we believe that Par6 phosphorylation by aPKC may be central to various extrinsic cues that can lead to the EMT. In addition to TGFβ stimulation, EMT has been shown to occur in response to ErbB2 receptor tyrosine kinase activation [15]. Indeed, Aranda and colleagues have shown that activation of ErbB2 leads to a complex formation between the ErbB2 receptor and Par6/aPKC and disrupts apical-basal polarity and tissue architecture. They further showed that inhibiting the interaction of Par6 and aPKC (using a Par6 mutant that does not bind aPKC) was required for this ErbB2 induced disruption of acinar formation in mammary epithelial cells [15]. Although Par6 S345 phosphorylation was not examined in this report, Par6 phosphorylation was later shown to be an important event in acinar disruption in murine mammary gland cells downstream of TGFβ [3]. Given the observations that both phosphorylation, and aPKC-Par6 interaction are important for acinar disruption and tissue organization in mammary gland epithelial cells, it would be interesting to investigate whether aPKC induced Par6 phosphorylation is a common theme in other tumour promoting pathways.

It is becoming increasingly clear that aPKC can execute its functions through phosphorylation events of interacting partners. Another interesting substrate of aPKC is Lgl, a member of the Scribble complex. First identified in D. Melanogaster, Scribble
complex proteins (which also include Scrib and Dlg) are localized to the basolateral regions of the cell and they help maintain normal junctional complexes and cell polarity [16-18]. Loss of Scribble complex proteins through genetic deletion results in a loss of cell polarity and growth control [16-18]. Interestingly, expression or mislocalization of these proteins is often observed in various carcinomas and all three members of the Scribble complex are considered tumour suppressor genes [18-20]. Importantly, active aPKC can phosphorylate Lgl to regulate its displacement from the apical domain, and in turn, Lgl inhibits aPKC function at the basolateral domain [21-25]. This mutual exclusion of aPKC and Lgl is important for tissue homeostasis, as cells overexpressing aPKC or depleted of Lgl leads to loss in cell polarity and hyperproliferation [23]. Strikingly, Lgl levels are reduced in various human solid tumours including human prostate, breast, ovary and lung [26] and reduced Lgl function correlates with reduced cell-cell adhesion [27]. Given the roles of both Lgl and Par6 phosphorylation by aPKC in cell-cell adhesions, it would be interesting to examine whether aPKC-Par6 phosphorylation plays a role in Lgl localization and activity during cancer progression – as it is likely that there is interplay between polarity complexes during the dissolution of tissue structure and tumour development.

A recent report has highlighted a role for another member of the polarity complex, Par3, in protecting against cancer progression [28]. Par3 which is an interacting partner of both Par6 and aPKC, as well as an inhibitor of aPKC kinase activity, has been reported to be a suppressor of breast cancer metastasis [28]. The authors report a down-regulation of Par3 in human breast cancer and that the loss in expression of Par3 inhibits E-cadherin junction stability, disrupts actin dynamics, and decreases cell-cell cohesion via a Tiam1-Rac-GTP pathway in mammary epithelial cells. Interestingly, knockdown of Par3 induces metastasis without an overt loss in E-cadherin or an increase in classical EMT phenotype (as measured by classical markers of EMT, snail and fibronectin). Instead, the loss of Par3 was observed to increase E-cadherin recycling from the membrane, thereby reducing the levels of stable and immobile E-cadherin at cell junctions which ultimately leads to a decreased cell-cell cohesion [28]. Our own results suggest that knockdown of aPKC increases basal E-cadherin levels in lung adenocarcinoma cells (chapter 3), and it would be interesting to explore E-cadherin recycling and junctional stability.
Interestingly, another report outlines a role for E-cadherin translocation during EMT through the endocytic adaptor protein Numb [29]. In epithelial cells, Numb was described to stabilize junctional complexes by binding to E-Cadherin and Par3 at adherens junctions [29]. However, upon stimulation with the EMT inducing growth factor HGF (hepatocyte growth factor), Numb dissociated from E-cadherin, and then sequestered the aPKC-Par6 complex to the plasma membrane and the cytosol. Both Par3 and E-cadherin were re-localized from the junction, leading to reduced cell-cell adhesion and the facilitation of EMT [29, 30]. Interestingly, aPKC has been shown to phosphorylate Numb to control integrin endocytosis at the leading edge of migrating cells, outlining an important role for aPKC in the normal endocytic function of Numb [31]. Given the recently described role for Numb in E-cadherin and Par complex subcellular localization and subsequently cell-cell adhesion and EMT, it would be interesting to examine whether Par6 phosphorylation directs the function of the endocytic adaptor Numb to control E-cadherin trafficking and recycling during EMT. Studying E-cadherin trafficking and recycling is particularly interesting since as mentioned earlier, aPKC can alter the trafficking, recycling and degradation patterns of membrane receptors [32, 33] and furthermore, Par complex proteins have already been implicated in membrane endocytosis and vesicular trafficking [34, 35]. Whether other polarity complex proteins regulate normal and/or oncogenic cellular processes through vesicular trafficking is a promising future area of study.

Thus, the Par polarity complex is considered to be involved in various cellular polarization processes that facilitate directional cell migration, apico-basal polarity and embryonic development. However, recent advances in our understanding of this complex reveal that these proteins can have different functions depending on the interacting partners, extracellular stimuli, and the cellular context in which the signalling occurs.

We have described above how the phosphorylation of Par6 leads to EMT, but Par6 phosphorylation has also been reported to regulate axon specification in naïve neurites [36], and invasive potential of epicardial cells [37]. Thus, the cellular outcomes of Par6 phosphorylation may depend on the cell type, subcellular localization, availability of binding partners and the combination of various extracellular and intracellular cues. How aPKC phosphorylation of Par6 plays a role in these multiple contexts is an area that
will require further investigation (Figure 5.2B). Specifically, we will likely require a better understanding of the signalling patterns of the Par interactome, and whether aPKC-Par6 phosphorylation alters the function and activation patterns of downstream effectors (and vice versa). Both Par6 and Par3 act as scaffolding nodes for various binding partners and effectors that allows this complex to elicit variable signals for multiple pathways. The factors that determine the binding and/or activation of specific GTPases, as well as the factors that control aPKC activity will likely be important in understanding how Par proteins regulate growth, organization, and the maintenance of cellular homeostasis, but also on cellular transformation and tumour progression.

Much work suggests that aPKC may be an attractive clinical target for protecting against tumour progression in certain contexts. In support of this idea, the loss of function experiments of aPKC in lung cancer cells reduces invasive activity and reduces tumour growth and expansion in vivo [38, 39] and a targeted small molecule inhibitor of aPKC-Par6 signalling is currently in early stage clinical trials for lung cancer [40-42]. Future work will uncover the intricacies of Par signalling pathways in normal and oncogenic circumstances. These new discoveries will undoubtedly help us further understand how polarity proteins control basic biological processes, as well as identify targets to block oncogenesis.
Figure 5.2

A

Epithelial Cells

Migrating Cell

B

What regulates aPKC-Par6 phosphorylation?

Extrinsic Stimuli
- TGFβ
- ErbB2?
- other regulators?

Intrinsic Stimuli
- GTPases
- Par3? (inhibitor?)
- other regulators?

Har6 interactions?

What does Par6 phosphorylation control?

- Front-rear polarization?
- Apico-basal polarity?
- Vesicular trafficking?
- Asymmetric cell division?
- Axon specification?
- GTPase activity?
- Loss of organization?
- Enhanced oncogenesis?
Figure 5.2. Roles for Par6 phosphorylation in EMT and Migration

(A) Proposed model for aPKC-Par6 induced plasticity. TGFβ-dependent Par6 phosphorylation in epithelial cells leads to junctional complex disassembly through Smurf1 mediated ubiquitination and degradation of RhoA. Degradation of RhoA leads to the depolymerization of filamentous actin (F-Actin) and loss of structural integrity of the cortical actin cytoskeleton, dissolution of junctional complexes, and subsequent reduction in cell-cell adhesion. Par6 phosphorylation may also regulate Rho-GTPases at the leading edge during cell migration. The aPKC-Par6 complex recruits Smurf1 to degrade RhoA at the leading edge to promote protrusive activity. Par6 phosphorylation has also been reported to specify axon differentiation, although the role of aPKC isoforms has not been defined.

(B) Major questions surrounding aPKC-Par6 phosphorylation. Understanding Par signalling will involve the investigation of the stimuli that control aPKC activation with respect to Par6 phosphorylation, how the Par interactome modifies and is modified by Par6 phosphorylation, and whether these factors contribute to homeostatic and oncogenic processes.
5.3 Limitations and Future Directions

The work carried out in this thesis was carried out in established cell lines using human recombinant TGFβ protein, and the majority of it was conducted to gain a mechanistic understanding of TGFβ signalling processes. Using cell lines provides a well characterized simplified platform that can be easily evaluated to understand the mechanistic biology of the TGFβ system. However, cell lines may not be fully representative of the results one might gather in an in vivo system. It would be interesting to test whether aPKC affects TGFβ induced EMT and apoptotic response using animal tumour models. Indeed, in line with our data, others have examined the roles of aPKC and Par6 in oncogenesis using mouse models [3, 38, 39] and have reported both aPKC and Par6 expression and activity are involved in tumour progression and EMT. However, how aPKC drives Par6 and or TGFβ mediated EMT in an in vivo setting is an area that deserves further exploration. Along with this, it would be interesting to examine human patient tumour samples and examine whether elevated P-Par6 levels correlate with aPKC expression levels. Furthermore, another limitation of our studies is that we have been studying these signalling pathways in 2-dimensional cell culture. Although this approach is widely used, analysis of EMT and tumour progression in 3D culture would also be a natural progression to understand the contributions of components of the ECM in our signalling system. It would be extremely interesting to look at epithelial cell acini formation and EMT in 3D cultures. Doing this while manipulating levels of aPKC and Par6 in the context of TGFβ signalling would further our understanding of the role of polarity protein dynamics in cell-cell junctional dynamics. Important contributions of the ECM and integrin signalling have been reported before with respect to aPKC and Par6 polarization processes [43] and thus it would be exciting to study whether Par6 phosphorylation by aPKC plays a role in acini formation and or disruption by TGFβ.

Another limitation of my studies is that I have not been assigning isoform specific functions between PKCα and PKCζ. However, whenever I could, I examined PKCα and PKCζ individually to assess their contributions to TGFβ effects. For example, in chapter 2, I knocked down each aPKC individually and examine P-Smad2 levels as well as receptor degradation, in chapter 3 I overexpressed each aPKC individually and examined
Par6 phosphorylation, and in chapter 4 I knocked down each aPKC individually and assessed P-p38 MAPK levels. Importantly however, knockdown of PKC\(\tau\) alone, resulted in compensatory expression of PKC\(\zeta\), necessitating the need for a double knockdown. In any case, for the most part, knockdown of each aPKC individually yielded similar responses in our analyses, and the double knockdown had the most robust effect. For example, in chapter 4, knock down of each aPKC individually increased p38 MAPK phosphorylation – but the double knockdown enhances p38 phosphorylation to the greatest degree. A similar trend was evident for the loss of E-cadherin. Thus, for the readouts that we examined, it seemed that both aPKC \(\tau\) and \(\zeta\) function redundantly. However, this does not mean that each of these PKCs is functionally redundant in a normally physiological setting. Although in our cell culture models we find they can execute some similar functions, possibly their tissue expression patterns and substrate availability may dictate their true individual function \textit{in vivo}.

Another limitation of my studies is that I often use overexpression to study protein-protein interactions. The reason I do this is primarily due to a lack of quality antibodies for immunoprecipitation of endogenous proteins, this is particularly true for the TGF\(\beta\) receptors and Par6. In any case, most of the interactions we have studied have been reported before. For example, in chapter 3, I showed that the interaction of aPKC\(\tau\) with the TGF\(\beta\) receptors occurs through Par6. A similar interaction has been reported before for aPKC\(\zeta\) [2]. The interaction between aPKC and the T\(\beta\)RI scaffolded by Par6 has been difficult to observe using endogenous proteins partly due to the lack of quality reagents, but also possibly related to my finding that aPKC reduces TGF\(\beta\) receptor complexes. I showed in chapter 2 and 3 that aPKC expression can reduce TGF\(\beta\) receptor expression, as well as displace Par6 from the complex. This suggests that aPKC binding may be involved in the controlled regulation of T\(\beta\)RI-Par6 complexes, making the detection of endogenous interactions challenging. Thus, future studies might include detecting the endogenous interaction of aPKC with TGF\(\beta\) receptors in the presence of a proteasome and/or lysosome inhibitor to test if that theory holds true. Furthermore, the utilization of \(1^{25}\) TGF\(\beta\) crosslinked to the receptors (as I utilized in chapter 2 for degradation studies) may also increase the sensitivity of the TGF\(\beta\) receptor signal
following immunoprecipitation of aPKC. Importantly, whenever possible I attempted to
immunoprecipitate endogenous proteins (for e.g in chapter 4, I precipitated endogenous
Smad2 to assess endogenous Smad4 or SARA association).

Another area in which I was limited was in the ability to assess TGFβ receptor
trafficking in human cells. Our lab used HAT cells (which are mink lung cells stably
expressing HA tagged TβRII) to study TGFβ receptor trafficking. This is primarily due to
a lack of antibodies for the TGFβ receptors sensitive enough to study using
immunofluorescence microscopy. Because the mink genome has not been sequenced, we
did not have the resources to knockdown aPKCs as we did in human cells. In the future,
it would be extremely interesting to conduct a detailed analysis of TGFβ receptor
internalization dynamics in aPKC knockdown cells. This may require the generation of
human cells stably expressing TβRII. Another approach would be to use biotinylated-
TGFβ, which would allow for the study of the internalization of activated TGFβ
receptors from the cell surface. Coupled with higher resolution confocal microscopy, one
could analyze receptor dynamics and study the residence time of activated receptors in
various intracellular compartments in aPKC knockdown cells (caveolae, early and late
endosome, and lysosomes) (Figure 5.3). This may shed light onto whether Smad2
dissociation from SARA occurs more efficiently at specific points in the endocytic
pathway, or whether TβRI-TRAF6 complexes are more likely to persist due to an
increased residence time in one particular intracellular compartment. Interestingly, the
shuttling ubiquitin binding protein p62 is known to bind both TRAF6 and aPKC to
control the intracellular trafficking dynamics and signalling potential of various receptors
[33, 44, 45]. Recent reports indicate that p62 acts as a signalling hub an can recruit and
oligomerize various signalling molecules to control cell survival, apoptosis, and protein
degradation and has important roles in cancer progression (reviewed in [46]). Using
overexpression models, in preliminary analysis I have found that p62 immunoprecipitates
with the TGFβ receptors (data not shown). It would be interesting to examine whether
p62 plays a role in the shuttling dynamics and intracellular itinerary of the TGFβ
receptors to control the pathways I have described in this thesis. This remains a
possibility as p62 is involved in the function of TRAF6 [44], as well as in lysosome
targeting of aPKC [33]. Examining whether p62 functions in the TGFβ pathway deserves further exploration.

All in all, a thorough and detailed analysis of TGFβ receptor intracellular trafficking dynamics in aPKC knockdown cells would shed light on how aPKC may be controlling signalling patterns. Furthermore, it is important to explore some of the mechanisms we have identified in an in vivo setting to confirm these findings in a more physiologically relevant model.
Figure 5.3. aPKC may control the subcellular itinerary of activated TGFβ receptor complexes to control specific TGFβ signalling outcomes

TGFβ receptors internalize into two distinct endocytic pathways. Clathrin-dependent internalization into the early endosome is important for propagating signals, whereas the caveolin-1-positive membrane raft compartment is involved in receptor degradation. aPKC plays roles in regulating the levels of TGFβ receptor complexes. This may be occurring through a co-ordinated signalling effort between aPKC and binding partners such as the late endosome sequestering protein p62, and the polarity protein adaptor Par6. Detailed understanding of how aPKC controls the intracellular itinerary of TGFβ receptors is not known (indicated by question marks). An analysis of how/whether aPKC controls these dynamic trafficking patterns through these intracellular compartments is warranted.
5.4 Conclusions

In conclusion, I have demonstrated that aPKC expression and activity are intricately linked to the control of multiple TGFβ pathways, including Smad2, Par6 and p38 MAPK. I have made the novel finding that the modulation of aPKC activity or expression alters the way non-small cell lung cancer (NSCLC) cells respond to TGFβ signalling by altering specific TGFβ-dependent pathways. I have provided mechanistic insight into how aPKC is involved in each of the mentioned pathways, as well as generated future avenues of research that deserve further exploration. Given the important roles of TGFβ signalling in developmental processes, cellular homeostasis, as well as the progression of cancer, continued understanding of the mechanisms which control how cells read TGFβ signals will further our appreciation of animal physiology in both the normal and diseased state.
5.5 References


## Appendix

### List of Inhibitors Used

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Reported Target and IC&lt;sub&gt;50&lt;/sub&gt; μM</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF 109203X (GFX)</td>
<td>PKC(IC&lt;sub&gt;50&lt;/sub&gt;): α(0.008) βI(0.018) ε(0.132) ζ(5.8)</td>
<td>[1]</td>
</tr>
<tr>
<td>Gö6976</td>
<td>PKC(IC&lt;sub&gt;50&lt;/sub&gt;): α(0.0023) βII(0.006) μ(0.02)</td>
<td>[1]</td>
</tr>
<tr>
<td>p38 MAPK Inhibitor</td>
<td>p38 MAPK (0.035)</td>
<td>[2]</td>
</tr>
<tr>
<td>MG132</td>
<td>Proteasome (0.1)</td>
<td>[3]</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Ribosome/translation (0.532)</td>
<td>[4]</td>
</tr>
</tbody>
</table>

Shown is a list of inhibitors used in these studies alongside some reported IC<sub>50</sub> values.


Curriculum Vitae

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